In vivo and *in vitro* studies on heavy doses and fluorescent-labelled vitamin C using ESR and fluorescence spectroscopy

Fabian Rozario

TU Kaiserslautern

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Dipl.-Chem. Fabian Rozario

Betreuer: Prof. Dr. Wolfgang Trommer

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For Penny, Joshua and Jasmine

Lack of ascorbic acid caused scurvy, so if there was no scurvy there was no lack of ascorbic acid. Nothing could be clearer than this. The only trouble was that scurvy is not a first symptom of lack but a final collapse, a premortal syndrome, and there is a very wide gap between scurvy and full health. But nobody knows what full health is!

Albert Szent-Gyorgyi, M.D., Ph.D.

Nobel Laureate

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Vorsitzender   : Prof. Dr.-Ing. S. Ernst

1. Berichterstatter   : Prof. Dr. W. E. Trommer
2. Berichterstatter   : Prof. Dr. H. K. Biesalski

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Abbreviations

ABTS.+	2,2`-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation
Ascoh	ascorbic acid
Ascr	ascorbyl radical
CABO	coronary aorta bypass operation
TEMP-COOH	3-carboxyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl
CPB	cononary pulmonary bypass
DHA	dehydroascrobate
DETAPAC	diethylenetriaminepentaacetic acid
DMAP	4-dimethyaminopyridine
DMPO	5,5-dimethyl-1-pyrrroline-N-oxide
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
HPLC	high performance liquid chromatography
ICAM	Intracellular adhesion molecules
VCAM	vascular adhesion molecules
MDA	moalondialdehyde
MANTA	6-O-(N-methylanthraniloyl) ascorbic acid
PBS	phosphate buffered saline
RDA	recommended dietary allowance
RNS	reactive nitrogen species
ROS	reactive oxygen species
TBARS	thiobarbituric-acid-reactive substances
XOD	xanthine oxidase

1   Introduction

1.1   Reactive oxygen species (ROS) and cellular damage

Oxygen is essential to life. The majority of intracellular oxidations of substrates results in the transfer of two-electrons to acceptors like NAD^+ or FAD, which are subsequently oxidized by the electron transport chain. The terminal step for the reduction of oxygen to water is catalyzed by cytochrome c oxidase, which binds to molecular oxygen where stepwise reduction of oxygen occurs without release of intermediates in the oxidation process. However, a stepwise one electron reduction is mostly favoured leading to the formation of oxygen radicals that can cause cellular damage.

The stepwise transfer of electrons to O_2 results in the formation of the following intermediates namely, superoxide anion ($O_2^{\bullet}$) the partially reduced hydrogen peroxide (H_2O_2), and hydroxyl free radical ($\cdot OH$).

The oxidative processes in cells generally result in the transfer of electrons to oxygen to water without the release of any intermediates, nevertheless a small number of oxygen radicals are inevitably formed due to the leakage in electron transfer reactions. The major source of intracellular oxygen radicals is the mitochondrial electron transport chain in which superoxide is produced by transfer of one electron to oxygen from the stable semiquinone produced during reduction of ubiquinone by complexes I and II of the electron transport chain. The oxygen radicals produced in the mitochondria include superoxide, hydrogen peroxide, and hydroxyl radical. ROS are also produced in peroxisomes; they oxidize fatty acids and other compounds by transfer of two electrons from $FADH_2$ to O_2 with formation of hydrogen peroxide, which is readily converted to the hydroxyl radical. The cytochrome p450 system localised in the ER can also produce oxygen radicals

ROS are also produced in cells during processes such as inflammation due to bacterial infection. To combat microbial infections, phagocytes produce and release toxic oxygen radicals to kill invading bacteria in a process known as respiratory burst. The phagocytes then engulf killed bacteria. However in prolonged infections, phagocytes tend to die, releasing toxic oxygen radicals into the surroundings causing damage to cells.

Cosmic radiation, ingestion of chemicals and drugs, as well as smog can lead to the formation of ROS. Damage to tissues due to ROS also occurs during perfusion with solutions containing high oxygen concentrations for patients during ischemia.

ROS can cause damage to all major classes of macromolecules in cells. The phospholipids present in plasma and organelle membranes are subject to lipid peroxidation. One significant consequence of lipid peroxidation is increased permeability of cell membranes and organelle membranes like mitochondria leading to an influx of Ca^{2+} -ions and with subsequent swelling of the cell leading to apoptosis. Amino acids are susceptible to attack by hydroxyl radicals resulting in fragmentation, cross-linking and aggregation of proteins. The most important consequence of ROS is the damage to both mitochondria and nuclear DNA leading to mutations. The binding of Fe^{2+} to DNA may result in localised formation of hydroxyl radicals that attack individual bases and cause strand breaks. Mitochondrial DNA is more susceptible to ROS damage, since the electron transport chain is the major source of toxic oxygen radicals.

Cells develop various mechanisms to remove ROS from their systems. Mammals have three different isozymes of superoxide dismutase that catalyses conversion of superoxide to hydrogen peroxide [Fridovich, 1995]. The cytosolic form of superoxide dismutase contains Cu/ Zn at its active sites, as does the extra cellular form of the enzyme (EC-SOD); however a unique mitochondrial form of superoxide dismutase exists with Mn at its active site. Hydrogen peroxide is removed by catalase, a heme-containing enzyme present at high concentrations in peroxisomes and to lesser extends in mitochondria and cytosol.

A major mechanism for the protection against the damage caused by ROS is gluthathione peroxidase, which catalyses the reduction of both hydrogen peroxide and lipid peroxides. This selenium-containing enzyme uses sulfhydryl groups of glutathione (GSH) as a hydrogen donor with formation of the oxidized disulfide form of glutathione (GSSH). Glutathione reductase converts the disulfide form of glutathione back to the sulfhydryl form using NADPH produced in the pentose phosphate pathway as an electron donor. Protection against ROS may also be gained from ingestion of oxygen scavengers such as vitamin C, vitamin E, and β-carotene [Hansford et al, 1999. Sohal, 1997].

1.2 Ascorbic acid and oxidative stress

1.2.1 Oxidative stress and antioxidant protection

Oxidative stress can result from increased production of reactive oxygen species and reactive nitrogen species (ROS/RNS). This imbalance between ROS/RNS and antioxidant defence, can result from numerous normal as well as pathophysiological conditions.

1.2.2 Ascorbate as an antioxidant

Vitamin C or ascorbate is known to be an essential antioxidant derived from the diet. Plant and animals can synthesize ascorbate from glucose in the liver or kidney. However, humans, other animals like guinea-pigs and fruit-bats lost the enzyme required for the terminal step (L-gulono-γ-lactone oxidase) and so require ascorbate to be present in the diet. This inability to make ascorbate is a universal inborn error of metabolism in humans.

Ascorbate is required as a cofactor for at least eight enzymes, of which the best known are proline hydroxylase and lysine hydroxylase, involved in the biosynthesis of collagen. Ascorbate is also required by the copper containing enzyme dopamine-β-hydroxylase, which converts dopamine to noradrenalin. Deficiency of ascorbate in human diet causes scurvy. Mammalian cells accumulate ascorbate from tissue fluids against a concentration gradient coupled to uptake of Na^+. Gut absorption of ascorbate is also Na^+-dependent. Several types of cells such as neutrophils take in dehydroascorbate through glucose transporters [Rumsey et al, 1997].

The structure of ascorbate resembles a pentose sugar with an en-diol ionizable group that allow the redox-chemistry characteristic for the molecule. These ionizable positions on the lactone ring have pK values of 4.17and 11.57, respectively. Therefore, ascorbate is largely present as a monovalent anion at physiological pH. One electron oxidation of ascorbate produces the ascorbyl radical, which is relatively unreactive. The poor reactivity of ascorbyl radical is the essence of many of ascorbate's antioxidant effects: a reactive radical interacts with ascorbate and a much less reactive ascorbyl radical is formed, which can be detected by EPR spectroscopy. Ascorbyl radical has pK values of 1.10 and 4.25 present as a monovalent

anion. Left to itself, ascorbyl undergoes a disproportionate reaction, regenerating some ascorbate and dehydroascorbate. In DHA, endiol hydroxyl groups on the lactone ring are replaced by keto groups. Consequently, the keto bonds are highly strained and are unstable. Eventually, the ring sturcture of dehydroascrobate is easily hydrolyzed to a linear molecule, 2,3-diketo-L-gulonic acid and further decomposed to oxalic and L-threonic acids (**Fig. 1**) [Bors and Buettner 1997].

The different ionized forms of ascorbate have different redox properties, so that the redox-chemistry of ascorbate is highly pH dependent. At higher pH, the rate of auto-oxidation with oxygen is much higher as a result of ascorbate dianion [Buettner, 1998]. Although most biological systems have a fixed physiological pH, some pH changes occur causing a shift in equilibrium.
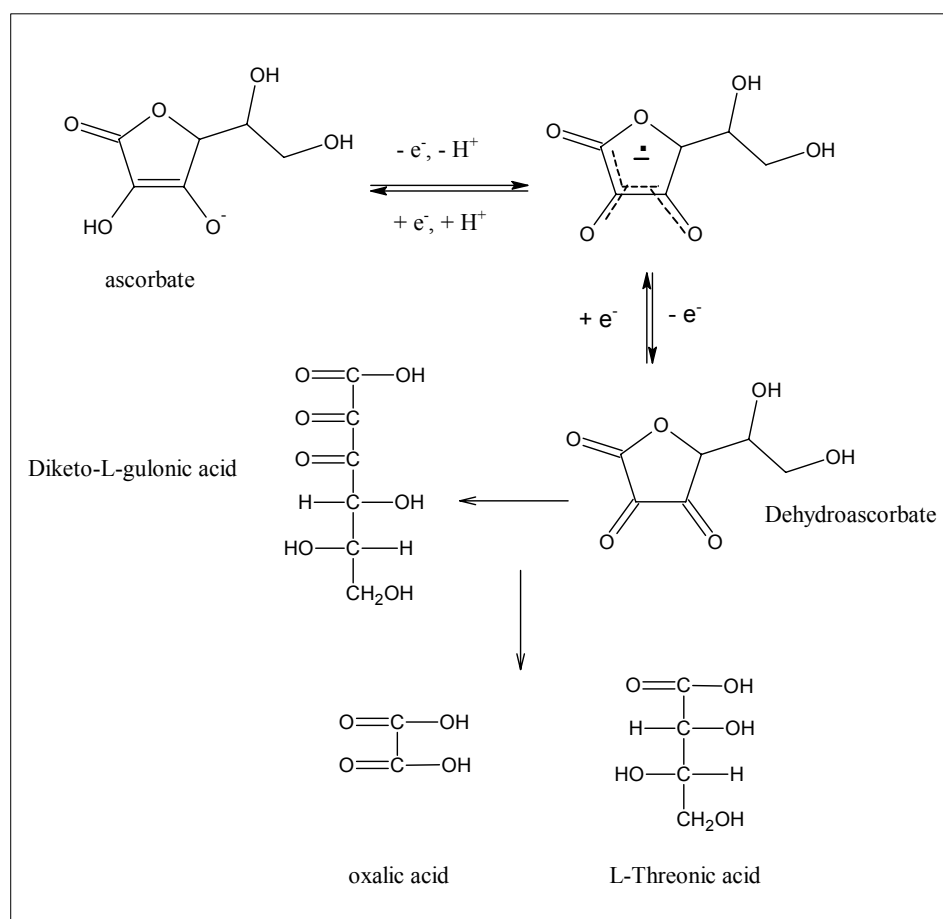
Figure 1 *Structure of ascorbic acid and its oxidation and degradation products. At physiological pH the acid form is largely ionised (ascorbate) since the pK_{a1} of ascorbic acid is 4.25.*

The chemistry of ascorbate shows that this antioxidant is an excellent reducing agent. Ascorbate can literally scavenge most of free radicals having greater reductions potential. Therefore, both thermodynamically and kinetically, ascorbate can be considered to be an excellent antioxidant [Buettner 1993]. Although ascorbyl radicals are formed from ascorbate oxidation with toxic free radicals, ascorbyl radicals does not react with oxygen to produce harmful peroxyl radicals. Ascorbyl radicals can by recycled by enzyme systems. Ascorbate can also help in recycling the lipophilic α-tocopherol radical (**Figure 2**) [Packer, Slater and Wilson 1979, Niki 1987]. *In vitro*, ascorbate has been shown to have a multiplicity of antioxidant properties, protecting various bio-molecules against damage by both reactive oxygen species (ROS) and reactive nitrogen species (RNS) [Buettner and Jurkiewicz 1996].
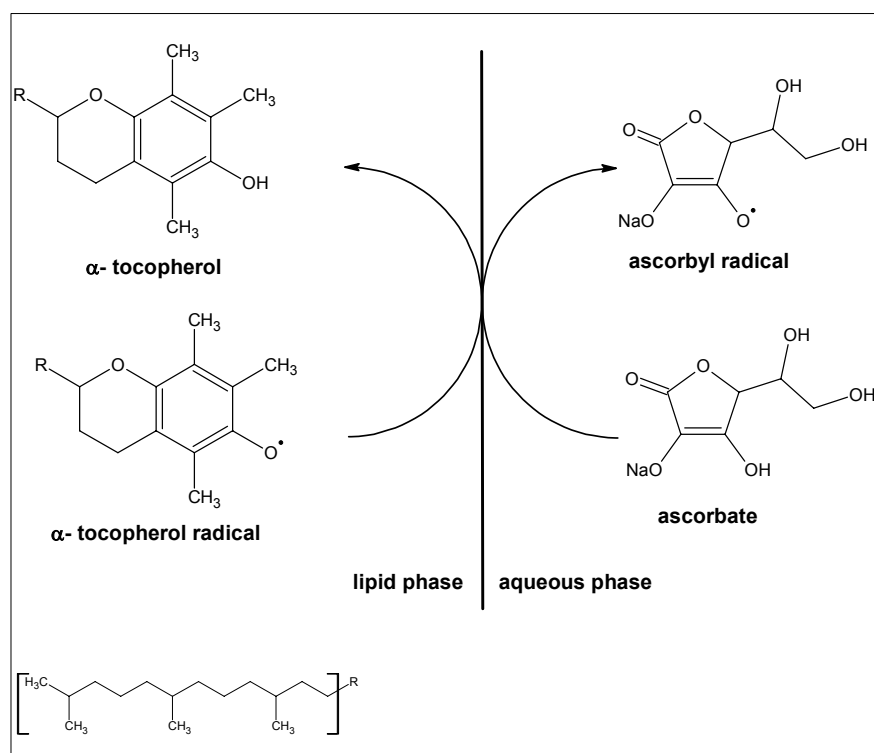
Figure 2   C and E recycling: *Interaction of ascorbate, with the α-tocopherol radical formed during lipid peroxidation, to produce ascorbyl radical which may eventually be regenerated* [Buettner 1993].

1.2.3   Ischaemia-reperfusion

Damage to the heart or brain by depriving a portion of the tissue of O_2 (ischemia) is a major cause of death in western society. Atherosclerosis, leading to the rupture of a lesion, thrombosis and the blockage of an essential coronary or cerebral artery is usually the culprit.

Severe restriction of blood flow, leads to very low oxygen concentrations (hypoxia), which can also result from a blocked artery [Bolli 1991].

1.2.4   Consequences of hypoxia

Hypoxic or ischemic tissues survive for variable time, depending on the tissue and the species it comes from. However, any cell made ischemic for a sufficient period (except erythrocytes) will be irreversibly injured. Tissues respond to ischemia in a number of ways [Halliwell and Gutteridge 2000].

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   ┌─────────────────────┐                    ┌─────────────────────┐
   │ deprivation of oxygen│                    │  xan. dehydrogenase  │
   └─────────────────────┘                    └─────────────────────┘
             │                                            │        Oxi. of SH-group
             ▼                                            │        and/or proteolysis
   ┌───────────────────────────────────┐                  │        (Ca²⁺- stimulated
   │ ATP → ADP → AMP →  adenosine       │                  │        protease
   └───────────────────────────────────┘                  ▼
           adenosine deaminase                 ┌─────────────────────┐
             │                                 │  xanthine oxidase    │
             ▼                                 └─────────────────────┘
          inosine                                         │
             │                                            │
             ▼                                            │
   ┌───────────────┐                                      │
   │  hypoxanthine │                                      │
   └───────────────┘         reperfusion restores O₂      │
             │                                            │
             └────────────────────┬───────────────────────┘
                                  ▼
                  O₂·⁻ , xanthine, urate, H₂O₂
                                  │
                                  ▼           Fe, Cu release  ◄─── ischaemic/ reperfusion
                                 ·OH                               tissue injury
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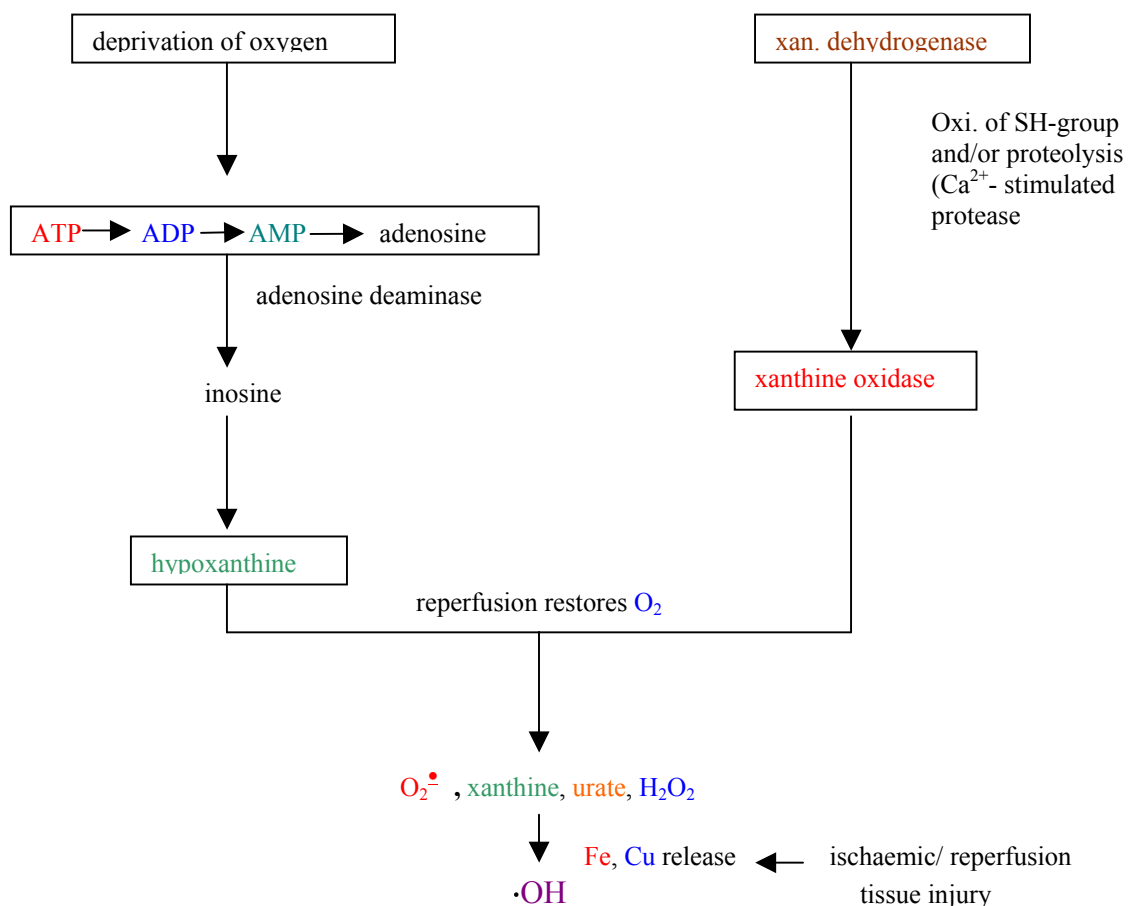
Flow chart 1 *A possible mechanism for tissue injury upon reoxygenation of ischaemic tissue* [McCord 1987]. *The enzyme that converts adenosine to inosine is adenosine deaminase. Adenosine modulates the activity of numerous cell types and may help to protect cells against damage cause by ischaemic-reperfusion.*

Early responses usually include increased rates of

- glycogen degradation and glycolysis leading to lactate production and acidosis
- ATP levels begin to fall
- AMP is degraded to cause an accumulation of hypoxanthine and
- Intracellular Ca^{2+} levels rise, activating Ca^{2+}-stimulated proteases and possibly nitric oxide synthase (NOS, if present)
- Membrane damage   (**Flow chart 1**)

1.2.5   Rexoygenation injury

If the period of ischemia or hypoxia is insufficiently long to injure the tissue irreversibly, much of it can be recovered by reperfusing the tissue with blood and re-introducing O_2 and nutrients. In this condition, reperfusion is a beneficial process overall. However, Parks et al showed in the early 1980s that re-introduction of O_2 to an ischemic or hypoxic tissue could cause further injury to the tissue (reoxygenation injury) that is, to some extent, mediated by ROS [Hearse and Bolli 1992]. The relative significance of reoxygenation (often called reperfusion) injury depends on the time of ischemia or hypoxia. If this is sufficiently long, the tissue is irreversibly injured and will die. Nevertheless, if a dying tissue is reperfused *in vivo*, this can release potentially toxic agents, such as xanthine oxidase and catalytic transition-metal ions, into the systemic circulation, causing injury to other body tissues. For example, gut ischaemia can lead to depression of heart function and xanthine oxidase can bind to endothelial cells.   It can produce O_2 to antagonize the action of NO, as well as generating potentially cytotoxic species such as $ONOO^-$ and H_2O_2 [Halliwell and Gutteridge 2000].

However, for a relatively brief period of ischemia/hypoxia, the reoxygenation injury component may become more important and the amount of tissue remaining undamaged can be significantly increased by including scavengers of ROS in the re-oxygenation fluid. The meaning of `relatively brief´ in this context depends on the tissue in question, whether one is dealing with ischemia or hypoxia and, if the latter, what degree of hypoxia was achieved.

Although the enzyme xanthine oxidase is frequently used as a source of O_2 in experiments *in vitro*, almost all xanthine –oxidizing activity present in healthy animal tissues is a dehydrogenase enzyme that transfers electrons not to O_2, but to NAD+, as it oxidizes

xanthine or hypoxanthine into uric acid. When tissues are disrupted, some of the xanthine dehydrogenase can be converted into xanthine oxidase by oxidation of essential- SH groups or by limited proteolysis (e.g. involving Ca^{2+} -stimulated proteases). Xanthine oxidase produces $O_2^{\bullet}$ as intermediates by the oxidation of xanthine or hypoxanthine [Schrier and Hess 1988]. The depletion of ATP in hypoxic tissue causes hypoxanthine accumulation. This hypoxanthine can be oxidized by the xanthine oxidase when the tissue is reoxygenated, causing rapid generation of $O_2^{\bullet}$ as intermediates and H_2O_2, which might lead to severe tissue damage. Released transition-metal ions can then promote ·OH formation (**Flow chart 2**) [Kehrer et al 1987].

1.2.6   Biochemical measures of oxidative stress

Free-radical species are highly reactive, short-lived and, as such, cannot be practically measured in human *in vivo* studies [Gutteridge and Halliwell 1990]. In the absence of a direct measure of free radicals, human studies have quantified the consequences of free-radical reactions employing methods that have significant limitations [Susanna et al]. To evaluate oxidative stress status, a wide array of methods is required to analyze antioxidants, enzymes, low-molecular-weight compounds with biochemical essays. The past decade has seen a real outburst for routine clinical evaluation of oxidative stress status leading to better understanding the harmful effects of oxidative stress [Sies 1991].

Measuring lipid peroxidation: Studies in the past were based solely on the determination of malondialdehyde as an *in vivo* marker of lipid peroxidation. Polyunsaturated lipids are very susceptible to free-radical attack. This process, referred to as lipid peroxidation, eventually yields several relatively stable decomposition products, including aldehyde compounds that can then be measured in plasma as an indirect index of free-radical activity [Gutteridge and Halliwell 1990]. Malondialdehyde, likely the most commonly measured index of oxidative stress in human studies, is only one of many aldehyde compounds produced by lipid peroxidation. Malondialdehyde is frequently measured in plasma by the thiobarbituric acid-reactive substances (TBARS) assay. Thiobarbituric acid reacts with malondialdehyde to produce a stable adduct that can be quantified using either spectrophotometry or high-performance liquid chromatography (HPLC). Although HPLC measures the thiobarbituric acid-malondialdehyde adduct more specifically that spectrophotometry, several other lipid-peroxide decomposition products and a variety of non-lipid related materials are also

detected[Janero 1990]. Furthermore, malondialdehyde arises from the degradation of a variety of non-lipid molecules, including proteins, carbohydrates, DNA, and bile pigments[Gutteridge 1981]. Therefore, although the TBARS assay is accepted as an index of oxidative stress, this method quantitates malondialdehyde-like material and does not specifically measure malondialdehyde or lipid peroxidation. Patients with coronary heart failure (CHF) and age-matched control subjects with normal LV (left ventriclar) function were studied[Mak et al 2000]. Many aldehyde products of lipid peroxidation in plasma were significantly elevated in the CHF patients. In summary, despite methodologic limitations, several studies [Belch et al 1991, McMurray et al 1993, Diaz-Velez et al 1996, Nishiyama et al 1997, Kieth et al 1998, Mallet et al 1998] have demonstrated an association between human CHF and elevated plasma aldehydes, the most commonly used marker of generalized oxidative stress.

Other biochemical assays for oxidative stress include:

8-hydroxy-2´deoxyguanosine (8-OH-dG): Reaction of ROS towards constitutive bases of DNA resulting in the transformation of guanine to 8-hydroxy-2`-deoxyguanosine. Accumulation of 8-OH-dG results in DNA mutations which may lead to cancer [Borek 1997].

Total antioxidant capacity (TAS): This test consists of a screening method that sums the various activities of all the antioxidant present in a biological medium. There exist several tests that differ by the ROS-generating systems used, the biological target to be oxidized and the chosen system for their detection [Ghiselli et al 2000].

Intracellular or vascular, adhesion molecules (ICAM, VCAM): Another alternative method in determining oxidative stress status is to observe ICAM and VCAM levels in plasma. Intracellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) are cell surface glycoproteins found on many cell types [Bevilacqua et al 1994]. Oxidative stress and expression of adhesion molecules, ICAM-1 and VCAM-1 are early features in the pathogenesis of atherosclerosis and other inflammatory diseases. Oxidation-reduction (redox) processes are known to regulate signal transduction leading to inducible ICAM-1 as well as VCAM-1 gene expression. Antioxidants have been suggested to have therapeutic potential in pathologies related to changes in cellular adhesion[Weber and Wolfgang 1996, Adam, Jessup,and Celermajer 1997].

Antioxidant vitamins: Vitamin E, vitamin C, and beta carotene remain the most widely studied antioxidants in the setting of large, randomized controlled trials. The largest of these

studies [Blot et al. 1993] has investigated the efficacy of antioxidant therapy in the primary prevention of cancer in 20,000 subjects. In contrast, only two small trials [Ghatak et al 1996, Keith et al 2001] of vitamin E therapy in patients with CHF were available. A non-randomized, uncontrolled, and unblended study of vitamin E supplementation in patients with CHF demonstrated improvement in markers of oxidative stress. However, no effect on quality of life was observed. Without positive evidence from clinical trials, encouraging the use of antioxidants based on the rationale that they are likely to be of no harm may be inappropriate. Large-scale trials of vitamin C, vitamin E, and beta carotene for the primary prevention of cancer [Blot et al 1993, Ghatak et al 1996, Hennekens 1998, Omenn 1996] and vitamin E in secondary prevention of acute ischemic coronary events [Stephens et al 1996, Rapola et al 1998] have raised important questions concerning the utility of antioxidant therapy. Although evidence for the role of oxidative stress in the genesis of both these conditions is more clearly established than it is for CHF, supplementation resulted in minimal or no clinical benefit. This may have related to the inability of available oral supplements to provide adequate antioxidant protection *in vivo* rather than an invalidation of the oxidative stress hypothesis. It may not be possible to attain physiologically effective concentrations in plasma with conventional oral regimens, especially in the case of vitamin C [Padayatty 2001].

For malignant disease, it may be necessary to intervene earlier and for a longer period of time. Of importance, the use of beta-carotene was not benign and associated with a significant increase in malignant disease [Omenn 1996]. These issues highlight the necessity of accumulating adequate clinical evidence prior to recommending the use of antioxidant vitamins for CHF.

1.2.7   Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass (CPB)

Cardiopulmonary bypass (CPB), a necessary and integral part of cardiac surgery, can induce deleterious effects, resulting in diffuse damage of several tissues. Although CPB is routinely performed without significant sequela, some patients can develop organ dysfunction involving kidneys, liver, lungs, CNS, or the cardiovascular system. Technical improvements achieved over the past years have contributed to the reduction of operative and postoperative mortality and morbidity.

The pathogenesis of these dysfunctions is multifactorial. It is believed to be triggered in part by a systemic inflammatory response to CPB, induced by the exposure of blood

elements to nonphysiologic surfaces [Bolli 1990]. As for heart damage, it could be more specifically associated with myocardial ischemia and reperfusion consecutive to cross clamping and clinically expressed as arrhythmia or "myocardial stunning," a depressed contractile function of major importance in the early postoperative period [Opie 1989, Lazzarino G et al 1994, Davies 1993]. Evidence suggests that ROS may play a significant role in the pathogenesis of these aforementioned phenomena. A systemic increase of various markers of oxidative stress [Ferrari R et al 1990, Morse et al 1998 Hearse and Bolli 1992] has been demonstrated to occur during CPB. Generation of oxygen free radicals could be the result of the activation of neutrophils occurring in response to an inflammatory reaction[Bolli 1998]. Moreover; it is known that ROS generation takes place during myocardial ischemia and reperfusion in various experimental models [Davies 1993] and in human heart. ROS could therefore be responsible for bypass-induced damages or impairment of myocardial recovery [Hearse and Bolli 1992, Curello et al 1995, Vergely et al 1998, Bendich et al 1986, Pietri et al1994].

1.3   ESR spectroscopy

1.3.1   Electron spin resonance and ascorbyl radicals

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) technique is based on the observation of unpaired electrons in a magnetic field brought into resonance with a microwave electromagnetic field.

An electron possesses a spin magnetic moment, so that in the presence of an applied magnetic field it can orient itself in a direction parallel or antiparallel (corresponding to the spin states α and β) to the field.

An electron spin makes the transition $\beta \rightarrow \alpha$ most efficiently when the energy separation of the two states matches the microwave frequency or the energy of the photon (in a magnetic field), for then the sample and electromagnetic field are in resonance.
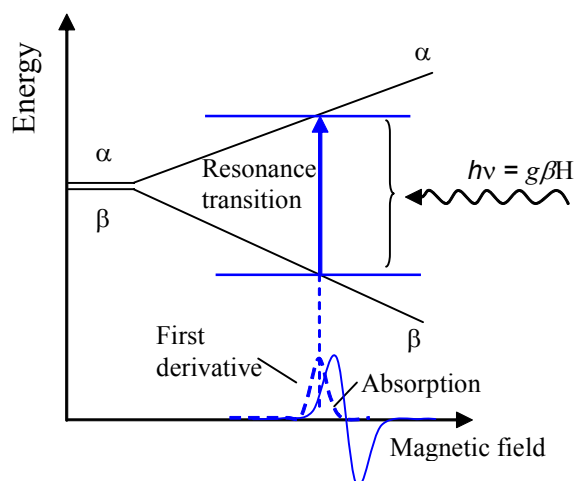
Figure 3 *Based on the fundamental theory of ESR. The energies of the α and β states of an electron diverge in a magnetic field. When the separation of the states matches the microwave irradiation, there is a strong resonant absorption.*

In continuous wave (CW) methods, the observation is performed by monitoring the absorption as the applied field is changed while the sample is exposed to a constant microwave radiation. But in pulse ESR spectroscopy, a short and intense microwave pulse is applied and the signals generated by the sample's magnetization are measured. By the application of Fourier transformation of the signal, a frequency spectrum from the sample is obtained.

The sample, which must be paramagnetic, may be a solid, a liquid or a gas. The peculiar appearance of the spectrum arises because of the detection technique, which is called **phase-sensitive detection** that monitors the first derivative of the absorption with respect to the field, so the point of zero slopes (where the plotted line passes through the horizontal axis) marks the peak of the absorption (**Figure 3**).

Three principal pieces of information are obtained:
- the position of the centre of the spectrum, as expressed by the *g*-factor,
- the shape of the spectrum, its hyperfine structure, and
- the width of the lines.

If the microwave frequency is v and the applied field is H the resonance condition is $hv = g\beta H$, where the *g*-factor is a parameter that takes into account the possibility that the local field is not exactly equal to H, β is a constant known as Bohr magneton. Measuring the

position of the spectrum enables *g* to be determined and then interpreted in terms of the electronic structure of the paramagnetic species.

Only ESR detects unpaired electrons unambiguously and yields incontrovertible evidence of their presence. In addition, EPR has the unique power to identify the paramagnetic species that is detected. EPR samples are very sensitive to local environments. Therefore, the technique sheds light on the molecular structure near the unpaired electron. Sometimes, the EPR spectra exhibit dramatic line shape changes, giving insight into dynamic processes such as molecular motions or fluidity.

The EPR spin-trapping technique, which detects short-lived, reactive free radicals, very nicely illustrates how EPR detection and identification of radicals can be exploited. This technique has been vital in the biomedical field for elucidating the role of free radicals in many pathologies and toxicities.

EPR spin-labelling is a technique used by biochemists whereby a paramagnetic molecule (i.e, the spin label) is used to tag macromolecules in specific regions. From the EPR spectra reported by the spin label, they can determine the type of environment (hydrophobicity, pH, fluidity, etc.) in which the spin label is located and local distance measurements can be studied [Loesel et al 1999. Wenzel et al 1974, Glöggle et al 1982, Fritzsche et al 1984, Reese &Trentham 1965].

Another important application for quantitative EPR is radiation dosimetry. Among its uses are dose measurements for sterilization of medical goods and foods, detection of irradiated foods, and the dating of early human artifacts.
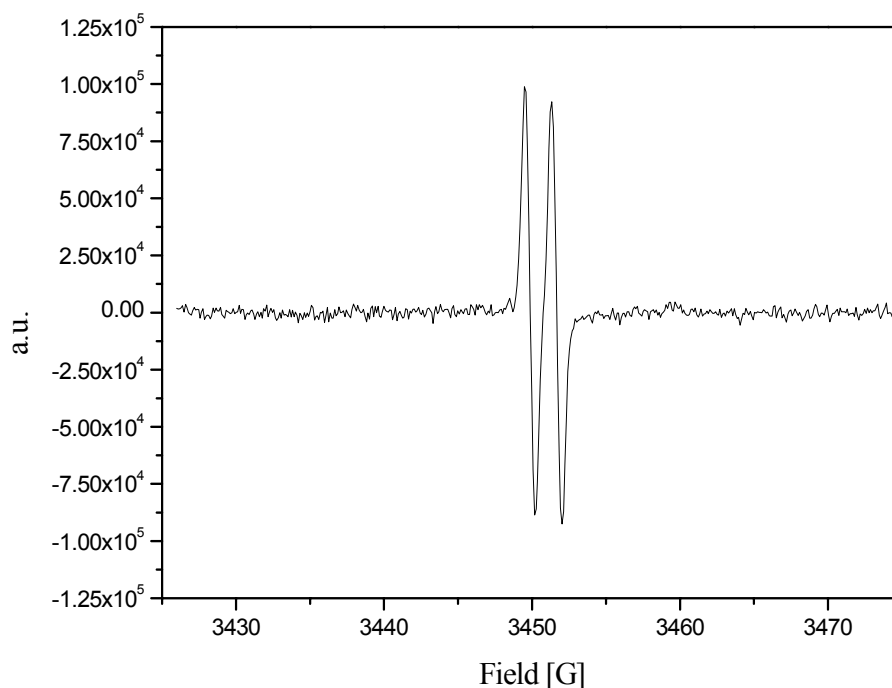
Figure 4 *ESR spectrum of the ascorbyl radical, usually seen as a doublet, oxidized from 5 mM ascorbate. Measured with a E580 spectrometer: 9.7 GHz, microwave power = 12 mW, modulation amplitude= 1G. Hyperfine splitting a^H = 1.8 G.*

An additional application of ESR spectroscopy is the determination and quantification of ascorbyl free radicals in living systems. The ascorbate anion is oxidized to produce the ascorbyl radical that can be detected and quantified by ESR methods. The ESR signal of ascorbyl radical is usually observed as a doublet (**Figure 4**), which increases proportionally during periods of oxidative stress. Thus, the ESR signal intensity of ascorbyl radical can serve as an indicator of oxidative processes taking place in biological systems.

In vivo, ascorbate behaves as a water-soluble antioxidant, reacting with reactive oxygen species [Bendich et al 1986]. This interaction yields AFR, and therefore the development of oxidative stress as a result of ROS. This can result in depletion of plasma and / or tissue ascorbate levels and at the same time increase of ascorbyl radical concentrations. Myocardial ascorbate consumption as a result of free radical reactions taking place during ischemia and reperfusion can be monitored in coronary effluents of isolated rat hearts by ESR detection of ascorbyl free radical. Ascorbyl radical concentration in plasma could be a reliable marker of

the occurrence of oxidative stress in patients undergoing aorta cross-clamping ischemia[Pietri et al 1994].

1.4   Fluorescence spectroscopy

The energy or electromagnetic radiation absorbed by a molecule when irradiated with light may be degraded into thermal motion or re-emitted. Light may be emitted at different time scales from an excited molecule, by processes called fluorescence or phosphorescence. Fluorescence decays immediately after the exciting radiation is removed. On absorption of light, a molecule is initially excited from the electronic ground state into the first excited state
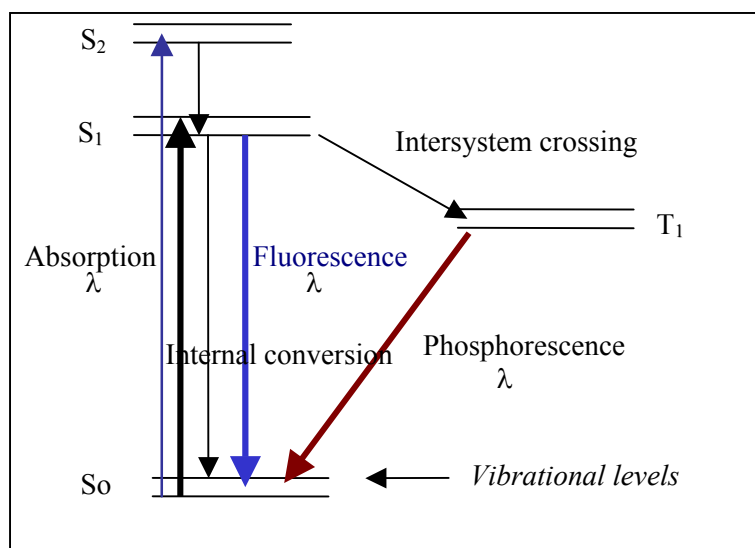
Figure 5 *Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption (⬆) and subsequent emission of fluorescence (⬇), phosphorescence (⬇) and internal conversion (↓).*

S1. This occurs so rapidly (about 10-15 s) that the nuclei in the molecule do not move during this process. The absorption of energy reduces the degree of binding in the molecule so that the distances between the nuclei are now too small. As a consequence, the molecule is not only in the electronically excited state, but also in a vibratory excited state. Over a time scale of about 10-12 s, the molecule relaxes to the vibrational ground state of the first electronically excited state S1. After a delay of a few nanoseconds in this state, which corresponds to the fluorescence life time, a photon is emitted in a process which lasts only 10-15 s. Following

emission of this photon, the nuclei are in a vibrationally or excited state in the electronically ground state S_0, and the molecule undergoes vibrational relaxation as before.

Because of these two vibrational relaxation processes that occur on absorption and emission, the energy of the emitted light is less than that of the absorbed light. Thus, for fluorescent groups, the wavelength of the emitted light is longer than that of the excitation light. Molecules that do not emit fluorescence disperse their energy in the form of non-radiative relaxation, or in the form of heat (internal conversion↓) (Figure 5). Fluorescent studies on its quenching properties may provide a useful tool to study competing reactions in solutions or biological systems.

Molecules in excited states can relax back to the ground state without any associated fluorescence emission. These nonradiative transactions may be due to intramolecular or intermolecular relaxation pathways, which quench the fluorescence. Quenching pathways compete with the fluorescence relaxation pathways and will reduce fluorescence emission. Some examples of nonradiative relaxation processes include resonance energy transfer, excited state chemical reactions, static quenching and collisional quenching. Resonance energy transfer is a process in which an excited donor molecule transfers its energy to a nearby acceptor molecule through near-field electromagnetic interaction. Static quenching occurs when a fluorophore forms a non-fluorescent complex with another molecule. Finally, collisional quenching is due to the loss of the fluorophore's excited energy state on collision with quenching agents (oxygen, metals, and paramagnetic molecules).

Fluorescence quenching can be a useful in fluorescence imaging. Resonance energy transfer imaging is a powerful tool for detection interaction and/ or co-localization of different moleculear species [Clegg 1995].

Intramolecular quenching can occur between co-valantely linked fluorophore-quencher pairs. Fluorescence and paramagnetic molecules as double sensors can be an important method for the determination of free radicals in biological systems. The energy transfer from a donor group (fluorophore) to an acceptor group (nitroxide) resulting in fluorescence quenching [Wu & Brand 1994, Bystryak et al 1986, Vogel et al 1994, Green et al 1990, Herbelin & Blough 1990]. Development of new double sensors containing fluorophore Dansyl derivatives and paramagnetic pyrrole-nitroxide derivatives can be introduced as indicators of oxidative stress taking place in biological systems [Tamas Kalai et al 2002].

2   Tasks and objectives

This thesis presents a study on the efficacy of heavy-dose vitamin C therapy as an anti-oxidant in healthy subjects and in patients undergoing aorta-coronary bypass operation. Correlations between oxidative stress and ascorbyl free radical are explored and compared with other biochemical parameters for oxidative stress. Ascorbate oxidations in *in vitro* systems are investigated and various aspects of ascorbyl free radical generated in these systems are reviewed. A new fluorescence labelled ascorbic acid is synthesized and information of its anti-oxidant capacity as well as intra-molecular fluorescence quenching is discussed. This thesis is to be concluded with the final chapter on problems faced in the quantification and evaluation of ascorbyl radicals. Also, optimization methods using ESR spectrometers for routine measurements are considered.

2.1   Biochemical studies of the efficacy of heavy-dose vitamin C supplementation on healthy subjects

The tolerance of heavy doses intravenous vitamin C therapy in healthy subjects is reviewed. Various aspects of ascorbate oxidation using ESR spectroscopy and dose-response kinetics are studied in relevance to heavy doses of vitamin C in healthy volunteers. Biochemical parameters by various markers of oxidative stress in correlation with ascorbyl radical concentrations after vitamin C supplementation are discussed. *In vivo* and *in vitro* kinetics of ascorbyl radicals in plasma may provide some information on the redox-chemistry of ascorbate at physiological pH.

2.2   Biochemical correlations of oxidative stress parameters from patients undergoing an aorta-coronary bypass operation after heavy doses vitamin C therapy

Oxidative stress triggered by ROS in patients undergoing coronary aorta bypass operation (CABO) is studied in correlation with ascorbyl radical concentrations in blood plasma. The efficacy of vitamin C as a water-soluble antioxidant to systemic inflammatory response and organ malfunction to patients undergoing CABO are explored. Moreover, intracellular and extracellular maintenance of ascorbate levels in reduced and oxidized forms are studied by

following their time-dependent changes. Thus, correlation between ascorbyl radical concentrations and ascorbate levels in plasma as a consequence of oxidative stress during surgery are investigated. In addition, results from various biochemical parameters for oxidative stress were compared with ascorbyl radical concentrations in order to investigate pro-oxidative aspects of heavy doses of vitamin C.

2.3 Chemistry and detection of ascorbyl free radicals compared to spin trapping techniques are studied *in vitro* as markers of oxidative stress.

Ascorbate, or vitamin C, is an important antioxidant, neutralizing oxidants and radicals against cellular damage. Generation of ascorbyl radicals by redox reactions and metabolism of ascorbate are reviewed using ESR spectroscopic techniques.

Various ionized forms of ascorbate show different redox properties so that the redox-chemistry of ascorbate is pH dependent. Therefore, ascorbyl radical concentrations at varying pH were measured with the help of ESR spectroscopy. Redox-chemistry of ascorbate involved in chemical as well as biochemical assays *in vitro* as source of oxygen radicals was investigated and ascorbyl radical generated in these systems was quantified.

In vitro ascorbate oxidation during hypoxia, anoxia and normal conditions in RKO cell lines are reviewed. In addition, ascorbate oxidation during reoxygenation is also investigated. Spin trapping techniques are introduced to confirm the results in relation to ascorbate oxidation.

2.4 Fluorescent labelling of ascorbic acid with N-methylisatoic anhydride

In order to study the dynamics of ascorbate in free radical generating systems, it would be appropriate to label ascorbic acid with a fluorescent marker. The two-faced character of fluorescent-labelled ascorbic acid can be determined using ESR and fluorescence spectroscopy. In the near future, *in vivo* and *in vitro* real-time measurements using fluorescence confocal microscopy to determine site related oxidative stress in living organisms might be of some significance. Therefore, the synthesis of a new fluorescence labelled ascorbic acid ester that possesses the characteristics of fluorescence emission as well

as showing paramagnetic properties was considered. Fluorescence quenching occurs between covalently bound fluorescent-quencher pairs. Therefore, fluorescent derivatives of ascorbate are expected to quench fluorescence when oxidized to ascorbyl radicals. This ability of detecting fluorescence as well as ESR signals could be useful in the identification of free radicals in living systems. A rather small fluorophore like N-methylisatoic anhydride was considered as a fluorescent label. Subsequently, redox properties of this new double-labelled ascorbic acid fluorescent ester are determined using fluorescence and ESR spectroscopic techniques.

2.5 Validation of routine ESR spectrometers with respect to the determination of the ascorbyl radical concentration in plasma samples in comparism with the Bruker ELEXSYS E500 Series spectrometer (E580)

In the course of this thesis a Bruker high-end research ESR spectrometer (E 500 Series) was employed for the determination and quantification of ascorbyl free radical concentrations in plasma. For routine measurements of plasma samples, low cost bench top spectrometers were to be tested and compared with the E580 spectrometer.

3   Results and discussion

3.1   Biochemical studies of the efficacy of heavy-dose vitamin C supplementation on healthy subjects

The importance of vitamin C, or ascorbate, in humans to protect the immune system, in collagen biosynthesis, and its antioxidant capacity are well known. Most of the animals can synthesize their own vitamin C from glucose except for man and certain animals and birds. Under stress situations animals (e.g. goat) can produce up to 7.5 g vitamin C per day, which may reveal the importance of this vitamin in the diet.

The dietary recommendation from the "2000 Deutsche Gesellschaft für Ernährung e.V." (DGE 2000) for vitamin C intake is about 100 mg per day for adults [Frei and Traber 2001]. The consumption of 100 mg/day is found to be sufficient to saturate the body pools for instance neutrophils, leukocyctes and other tissues [Carr and Frei 1999]. However, from a therapeutic point of view [Levine et al 1996], the dietary recommendation of vitamin C was even suggested to reach in grams, since stress, smoking, infections and burns deplete the ascorbic acid reserve in the body and demand higher doses of ascorbic acid supplementation.

There are also speculations about the benefits of higher vitamin C doses. Cu^{2+} and Fe^{3+} ion-ascorbate mixtures stimulate free radical damage in DNA, lipids and proteins *in vitro* [Halliwell and Gutteridge 1990, Porter 1995, Podmore et al., 1998, Jenner et al, 1998]. Nevertheless, *in vivo* studies show that metal ions are not freely available [Bendich et al 1986, Levine 1986] but bound to proteins like transferrin, haemoglobin and ferretin. The pro-oxidant activity of vitamin C in the presence of metal ions underlies its antioxidant protection in lipid peroxidation with or without iron supplementation. Current evidences also suggest the protection of ascorbic acid against atherogenesis by inhibiting LDL oxidation. The beneficial effects of ascorbic acid in collagen biosynthesis and its antioxidant property may play a vital role in the eradication of many diseases in humans.

To facilitate an overview on oxidative stress and anti-oxidant defence mechanism of ascorbate, healthy volunteers were intravenously given very high doses of vitamin C (Pascoe GmbH as Vitamin C-Injektopas®) containing 750 mg or 7.5 g ascorbic acid. Infusion therapy of vitamin C allows higher dosage, since oral administration of more than 200 mg may prevent resorption through epithelial walls.

Serum levels of vitamins C and E as well as biochemical parameters indicative of oxidative stress including thiobarbituric-acid-reactive substances (TBARS), malondialdehyde (MDA)

and 8-oxoguanine as determined at the University of Hohenheim[*] were correlated with the ascorbyl radical concentrations.

3.1.1 Protocol of healthy subjects with intravenous infusion of 7.5 g and/ or 750 mg vitamin C

Six healthy male volunteers, between 18 and 55 years old, were intravenously injected with either 7.5 g or 750 mg of vitamin C daily for six days. After two weeks washout phase the same volunteers received the alternate amounts, 7.5 g or 750 mg respectively, again for six days. The healthy subjects were divided into four groups and were kept under observation for over 29 days.

3.1.2 Classification of the clinical parameters

Primary parameters: Ascorbate, and ascorbyl radical, concentrations in plasma.

Secondary parameters: Malondialdehyde (MDA), and vitamin E levels in plasma. 8-Oxoguanine, 8-oxoadenine, oxalate and calcium levels in urine.

3.1.3 Primary Parameter: Determination of ascorbyl radicals in blood plasma of healthy subjects using ESR spectroscopy

After intravenous administration of 750 mg or 7.5 g vitamin C in healthy volunteers, blood was withdrawn before and 0.5, 1, 2, 4 and 8 hours after infusion. Ascorbyl radical concentrations in EDTA-plasma were determined using ESR spectroscopic methods. Ascorbyl radical concentrations were determined by double integration of the ESR spectra with a stable nitroxide radical such as 3-carboxyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl as a standard.

* Institut für Biologische Chemie und Ernährungswissenschaft an der Universität Hohenheim. Silke Mrosek et al.
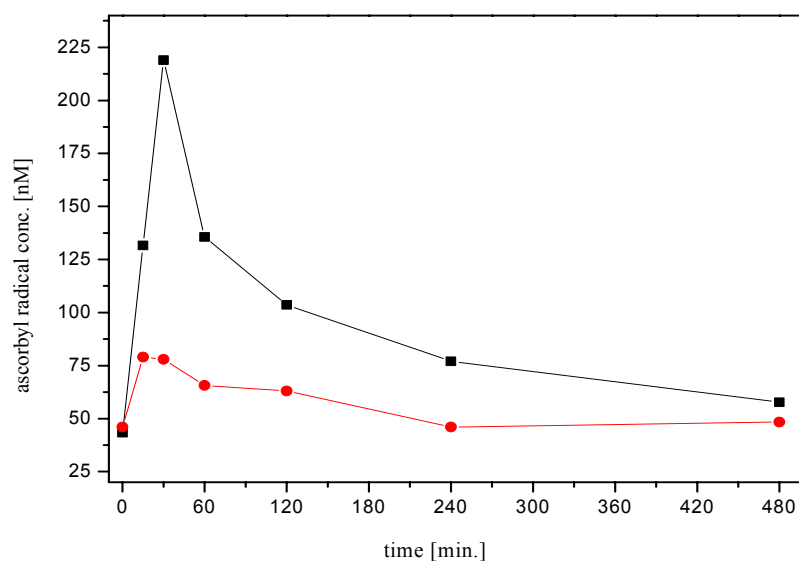
Figure 6 *Average ascorbyl radical concentrations in blood plasma of 6 healthy subjects after vitamin C infusion of 750 mg (–●–) and 7.5 g (–■–) on day 1 and day 23. Ave. stdev. 7.5 g = ±41, ave. stdev. 750 mg = ±23.*

Dose-response curves of ascorbyl radical concentrations in volunteers treated with vitamin C initially showed an increase in the concentrations (**Figure 6**). Maximum radical concentrations were seen after 30 minutes of vitamin C infusion. However, ascorbyl radical concentrations decreased steadily after 30 minutes with half-life of 70-100 minutes. The rate of free radical degradation slowed down through the last 8 hours after vitamin C infusion. Healthy subjects administered with 7.5 g vitamin C showed a 3-fold increase in the ascorbyl radical signal in the ESR spectrum in relation to subjects after 750 mg vitamin C infusion.

What do the dose-response curves of ascorbyl radical concentrations during a time span of 8 hours in healthy subjects after vitamin C infusion suggest? In order to pursue the dose-response curves of ascorbyl radicals, it is first necessary to understand the chemistry of ascorbate oxidation. As seen in **Figure 1**, oxidation of ascorbate usually occurs in a two-step reaction resulting in the formation of ascorbyl radicals and dehydroascorbate. Moreover, two molecules of ascorbyl radical can disproportionate forming one ascorbate and one dehydroascorbate molecule, respectively.

The presence of the en-diol group in the molecule makes it a member of a redox system possessing electron donating and accepting properties. Is it possible that the formation of ascorbyl radicals in plasma occurs as a consequence of ascorbate oxidation? Although,

reactive intermediates during oxidative phosphorylation have been sequestered in protein complexes, some radical intermediates leak out during regular mitochondrial metabolism. Mostly, this may take place when iron proteins ferritin and transferrin that are supposed to sequester free irons are damaged [Clark and Pearson 1989]. Therefore, free iron present in the system becomes available in free radical reactions causing injury to tissues. This indicates that ascorbate oxidation does not end at the level of ascorbyl radical, but that it continues to DHA within a short time interval. Consequently, ascorbyl radical signals decrease after reaching a maximum at early time points. Therefore the reaction kinetics of ascorbate oxidation in biological systems and determination of their respective concentrations may prove to be rather complex.

Under oxidative stress, the consumption of ascorbate can be high, and without regeneration, ascorbate would soon be depleted. Apparently, volunteers after vitamin C infusion showed similar non-linear dose-dependent changes in ascorbyl radical concentrations. Nevertheless, it should be noted that, divergences at higher ascorbyl radical concentrations within healthy subjects were statistically significant with standard deviations of 80 ± 33 for subjects after 750 mg vitamin C and 219 ± 103 for patients after 7.5 g vitamin C dosage, at 30 minutes. Large error bars and variability in their mean values can possibly be attributed to individual disparities at peak concentrations. Although numerous studies on recommended vitamin C intake was mostly attributed to gastrointestinal tract absorption by oral supplementation [Levine et al 1999, Vojdani et al 2000], the results presented here were determined from healthy subjects using vitamin C infusions therapy.

3.1.4 Stability of ascorbyl radicals in plasma under laboratory conditions at physiological pH in the presence of anticoagulant and ferrous irons

Ascorbate is largely present as a monovalent anion at physiological pH so that the redox-chemistry is highly pH dependent. Though most biological systems have a fixed physiological pH, some variations may occur in tissues and under certain pathological conditions. Moreover, during regular clinical procedures blood is withdrawn with monovettes containing EDTA as anticoagulant, which may influence plasma pH. Although ascorbyl radical has a relatively long half-life stabilized by resonance of the conjugated bonds, pH and oxidants can largely influence its stability.
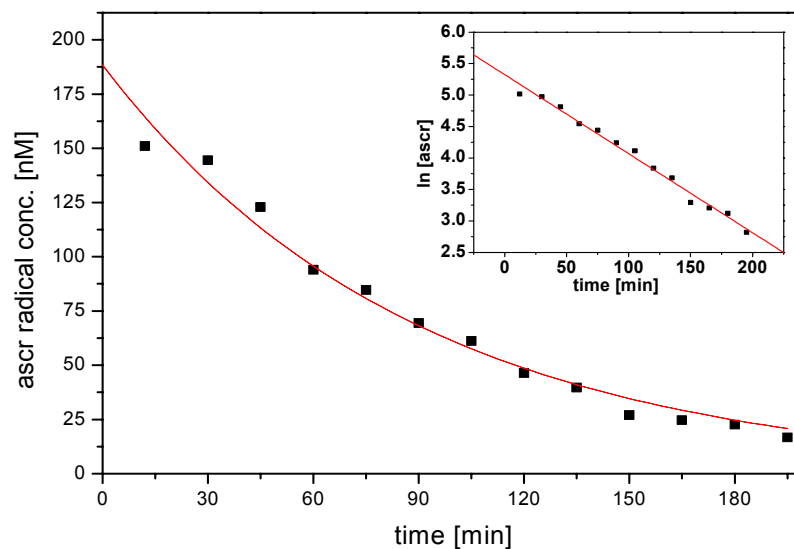
Figure 12 *First order exponential decrease of ascorbyl radical concentrations* (—■—) *in blood plasma plotted against time from healthy subjects after the infusion of 750 m g vitamin C. The red line is a mono exponential fit with a half-life (τ) of 86 ± 4 minutes. Inset: Verification of first-order equation by plotting ln [ascr] versus time.*

To test this, the stability of ascorbyl radicals in blood plasma containing EDTA was investigated under regular laboratory conditions. Kinetics of ascorbyl radical was measured at 3 different concentrations. ESR measurements were repeated successively on the average at every 15 minutes until no significant changes in ascorbyl radical concentrations were observed. The data presented in **Figures 12** and **13** shows a time-dependent exponential decay of ascorbyl radicals that reached equilibrium after 3 hours. Therefore, a non-linear curve fit was applied for all ascorbyl radical levels by assuming the reaction is first-order (**Figure**s **12**, **13** and **14**). To verify first-order kinetics, logarithmic plots of ascorbyl radical concentration versus time showed linearity with less than 10% -deviation. Graphical data from the 3 different ascorbyl radical concentrations yielded straight lines confirming first-order kinetics. Nevertheless, higher ascorbyl radical concentrations tend to show shorter half-life decay rates (**Figure 13**). This is in clear contrast with first-order kinetic rate laws as seen in **Figure 12** and **Figure 14**.
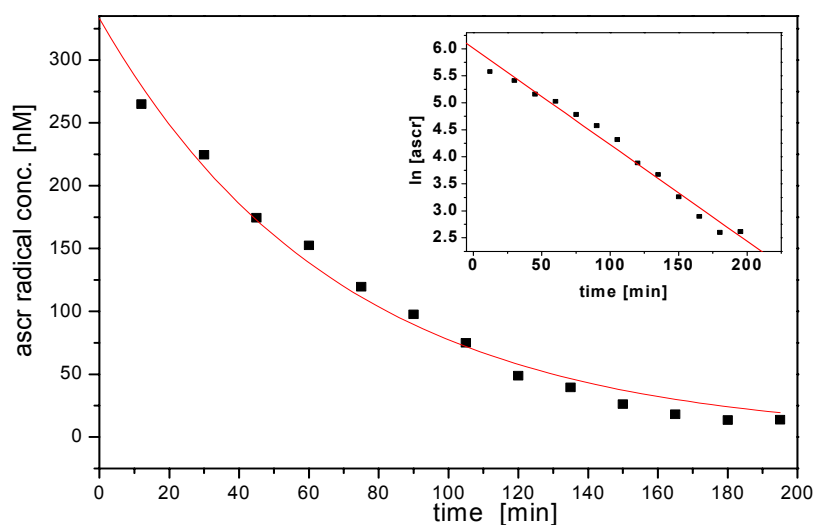
Figure 13 *1-order decrease of ascorbyl radical concentrations in blood plasma plotted against time from volunteers after the infusion of 7.5 g (—■—) vitamin C, $\tau =69 \pm 3$ minutes. Inset: Verification of first-order equation by plotting ln C_{ascr} versus time.*
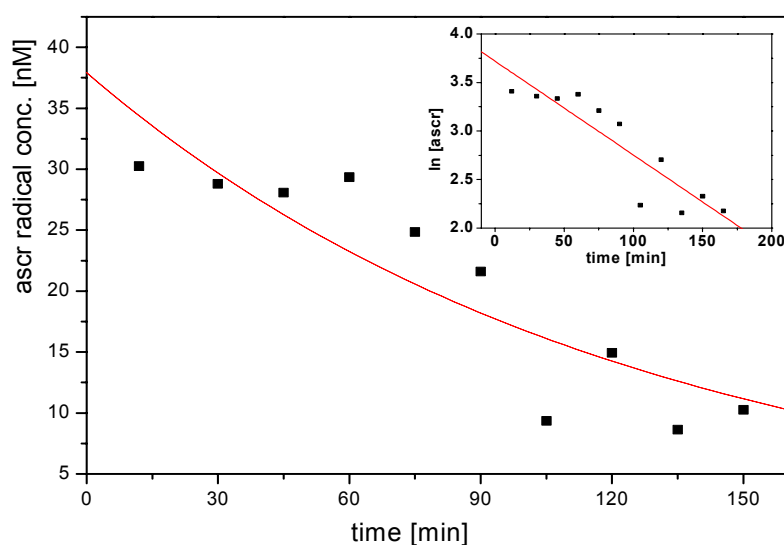
Figure 14 *First order exponential decrease of ascorbyl radical concentrations in blood plasma from volunteers without vitamin C infusion (—■—), plotted against time. $Y = Y_0 +A_1 e^{-x/\tau}$, $\tau =123 \pm 22$ minutes. Inset: Verification of first-order equation by plotting ln C_{ascr} versus time.*

A further step taken to study the kinetics of ascorbate oxidation is to establish the dose-response behaviour of the ascorbyl radical decay *in vivo* and to compare it with *in vitro* (**Figure 15**). Ascorbyl radical concentrations *in vitro* were determined in plasma withdrawn from healthy volunteers and measured at regular laboratory conditions. Decomposition of ascorbyl radical concentrations showed non-linear exponential kinetics with a half-life of 75 minutes *in vivo* and a half-life of 55 minutes *in vitro*. These results reveal *in vivo* redox reactions occurring in normal metabolic processes in which, ascorbate is being recycled. In contrast, *in vitro* decomposition of ascorbyl radical in plasma occurred mainly as a result of disproportionation.
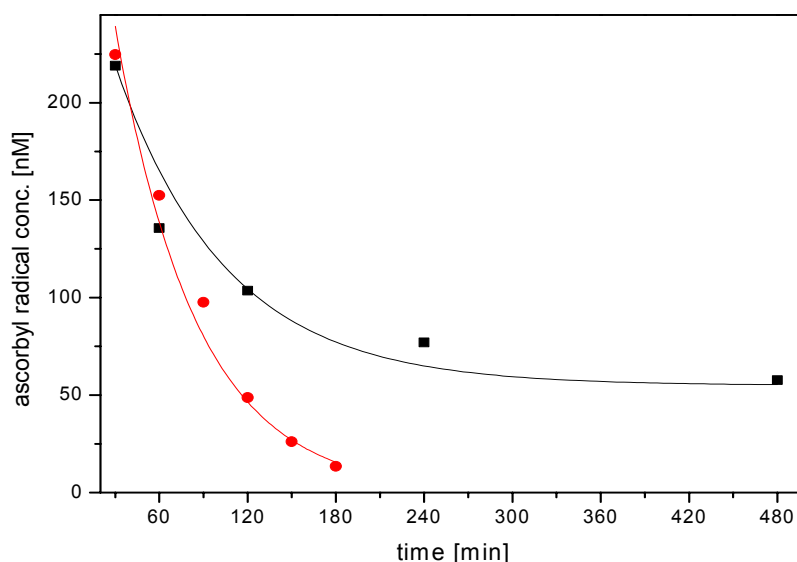
Figure 15 *The correlation between the decrease of ascorbyl radical concentrations in blood plasma of a healthy volunteer (–■–) and the decrease of radical concentration in blood plasma after the withdrawal of blood at laboratory conditions (–●–).*

In general clinical procedures, venous blood drawn by Lavender Top containing EDTA as anticoagulant are centrifuged and haemolysed plasma samples decanted in eppendorfs and are immediately shock-frozen. Prior to the ESR measurements, these probes are once again thawed in warm water. This repeated freezing and refreezing of plasma usually did not influence the ascorbyl radical signal intensity (**Figures 16** to **18**). However, an increase in signal intensity was detected in some of the probes. A plausible explanation could be the improper handling of blood plasma after the withdrawal of blood, in which few erythrocytes leak into the plasma and accelerate the oxidation process in the presence of free metal ions.
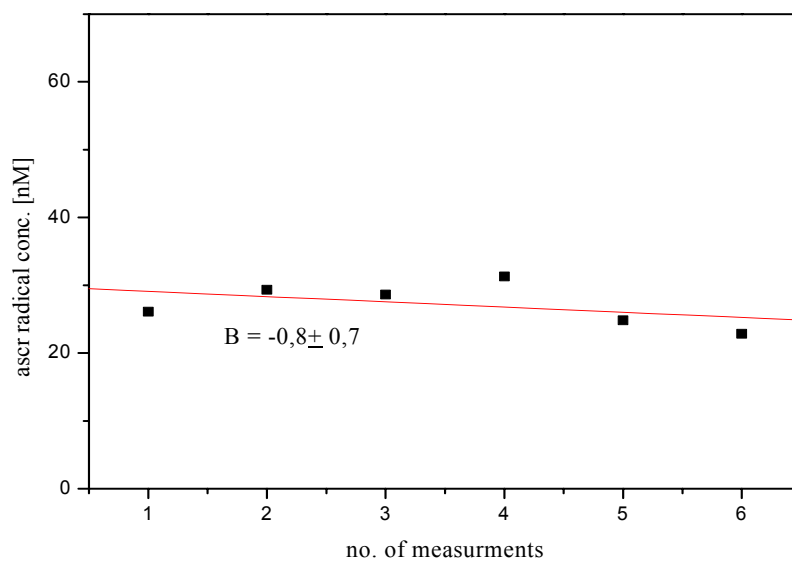
Figure 16 *Ascorbyl radical concentrations of repeatedly shock- frozen and thawed blood plasma. Linear fit applied for a radical concentration of 26 nM B= -0.8 ±0.7.*
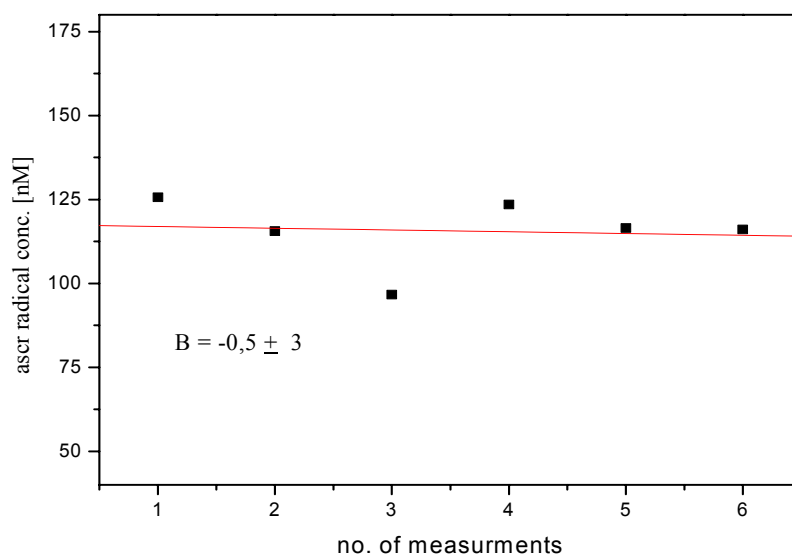
Figure 17 *Ascorbyl radical concentrations of repeatedly shock- frozen and thawed blood plasma. Linear fit applied for a radical concentration of 126 nM, B= -0.5 ± 3.*
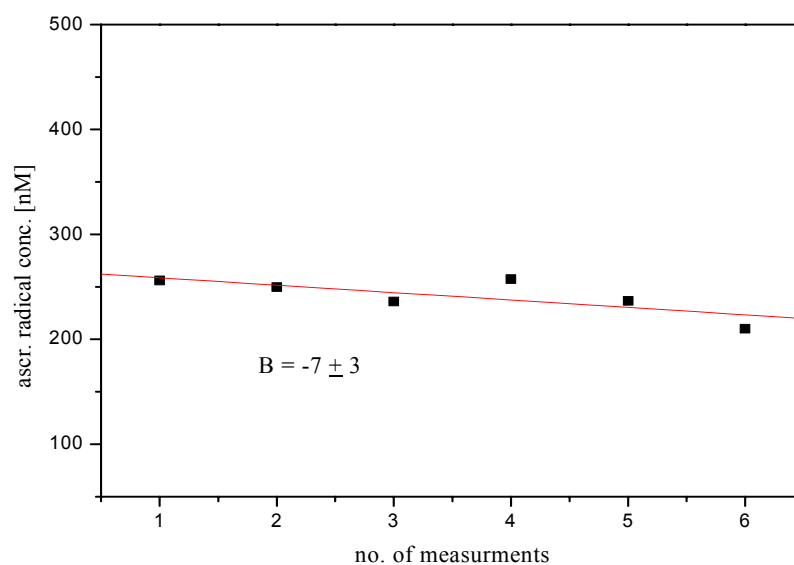
Figure 18 *Ascorbyl radical concentrations of repeatedly shock- frozen and thawed blood plasma. Linear fit applied for a radical concentration of 256 nM, B= -7 ± 3.*

To prove that traces of metal ions in plasma can influence ascorbyl radical concentrations, ascorbate oxidation in plasma was studied with and without the addition of free metal ions as seen in **Figure 19**.
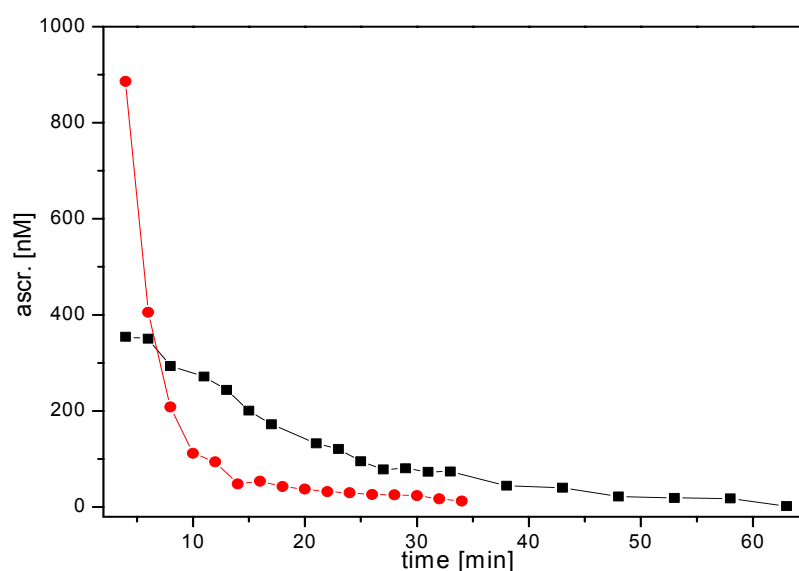
Figure 19 *Changes in ascorbyl radical concentrations after the addition of 24 μM Fe^{2+} (—●—) in plasma containing 2.4 mM ascorbate as compared with EDTA plasma (—■—).*

Presence of ferrous ions in plasma caused a drastic increase in ascorbyl radical signal intensity initially. However, the presence of plasma EDTA in moderate concentration did not largely influence ascorbate oxidation. The time-dependent decrease in ascorbyl radical concentrations as compared to untreated plasma showed the involvement of the ferrous iron-catalyzed oxidation of ascorbate. Ascorbyl radical concentrations treated with ferrous irons in plasma decreased by at least 50 % in 5 minutes. The presence of EDTA in the monovettes still allows for metal-dependent oxidation due to the remaining traces of the metal in plasma (**Figure 19).**

It has been suggested that DETAPAC proved to be a better metal chelator as compared to EDTA[Buettner and Jurkiewicz 1996]. Therefore, changes of ascorbyl radical concentrations against time were investigated in the presence of EDTA and DETAPAC (**Figure 20**). Both of the metal chelators reduced the rate of free-radical oxidation of ascorbate as compared to the plasma containing PBS. However, DETAPAC in the plasma reduced the rate of ascorbate oxidation considerably. These results showed that DETAPAC is considered a better choice of a chelating agent in studies of ascorbate oxidation in human plasma. Also, the initial ascorbyl radical concentrations of plasma containing DETAPAC were much lower than in EDTA plasma.
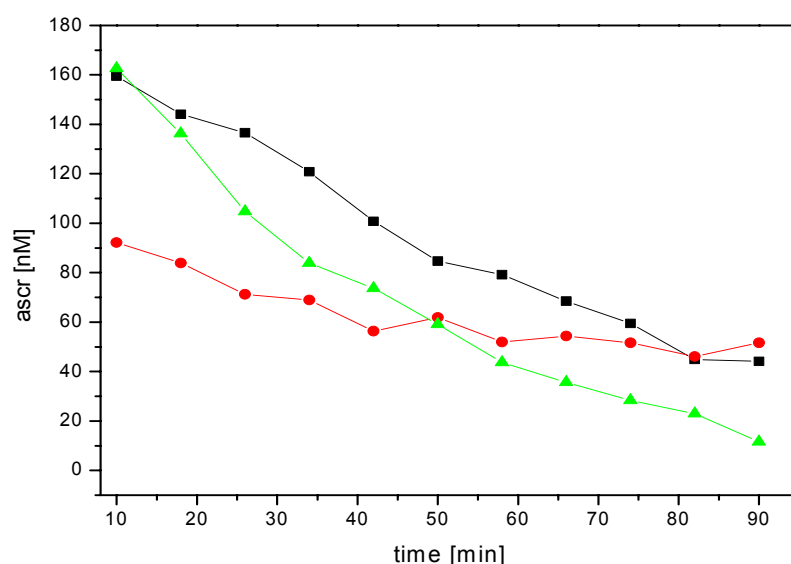
Figure 20 *Influence of metal-chelators such as EDTA (—■—) and DETAPAC (—●—) on the free radical oxidation of ascorbate in plasma at physiological pH under regular laboratory conditions in relation to PBS plasma (—▲—).*

Ascorbyl radical formation by ascorbate oxidation is also dependent on pH (**section 3.3.1**). Although the experiments performed in this study were done at physiological pH (7.4), plasma left standing for some time at laboratory conditions altered its pH. Though the pH changes in plasma were small, they influenced the rate of ascorbate oxidation considerably. To prove this, studies on ascorbate oxidation to free radicals were performed at two separate pH values. **Figure 21** show that an increase in plasma pH by 1 unit raises the ascorbyl radical concentration by at least 140 %.
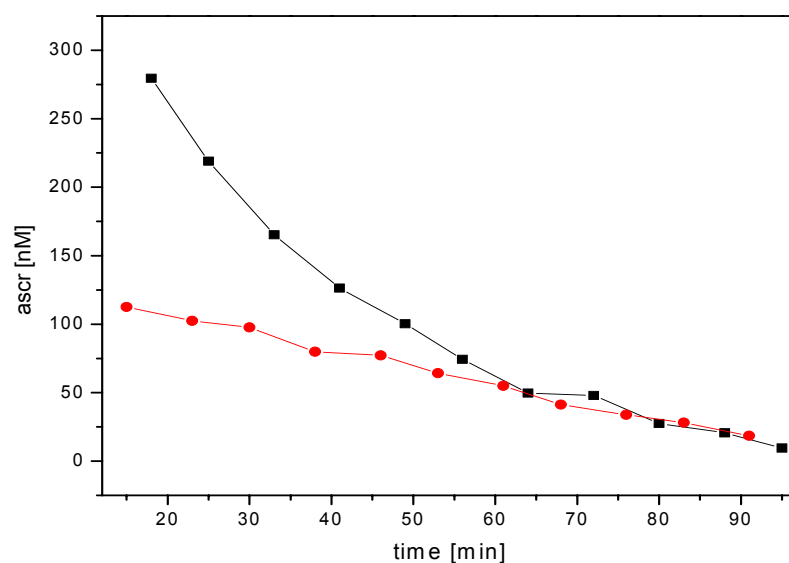
Figure 21   *Changes in ascorbyl radical concentrations in plasma at pH 7.64 (–●–) and pH 8.63 (–■–).*

Subsequently, the effect of plasma pH on ascorbyl radical intensities during time-dependent measurements was investigated. The Data in **figures 22** and **23** show that the decreases in ascorbyl radical concentration cross correlates with increasing pH in plasma. This increase in plasma pH, although very small, obviously plays an important role in studies of ascorbate oxidation kinetics.
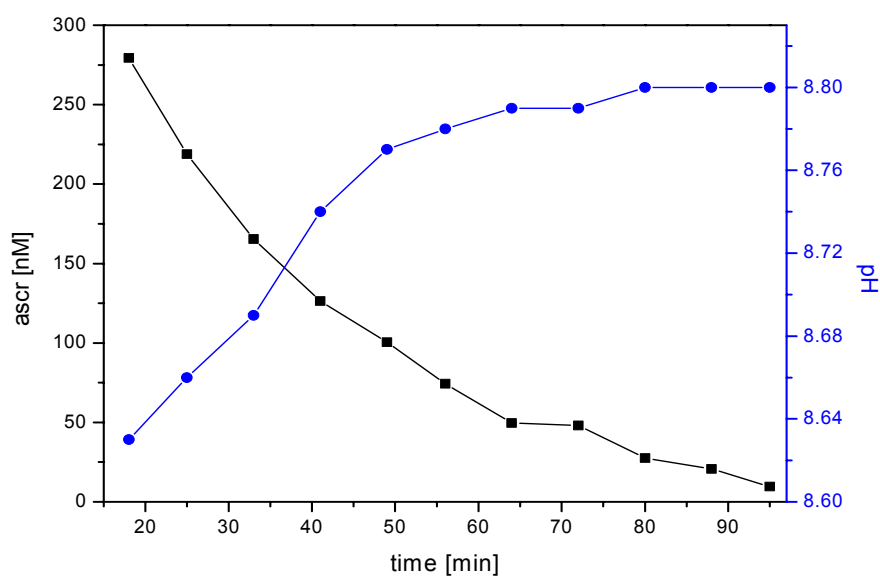
Figure 22   *Time-dependent cross correlations between ascorbyl radical concentrations (—■—) and the pH (—●—) of plasma measured under laboratory conditions.*
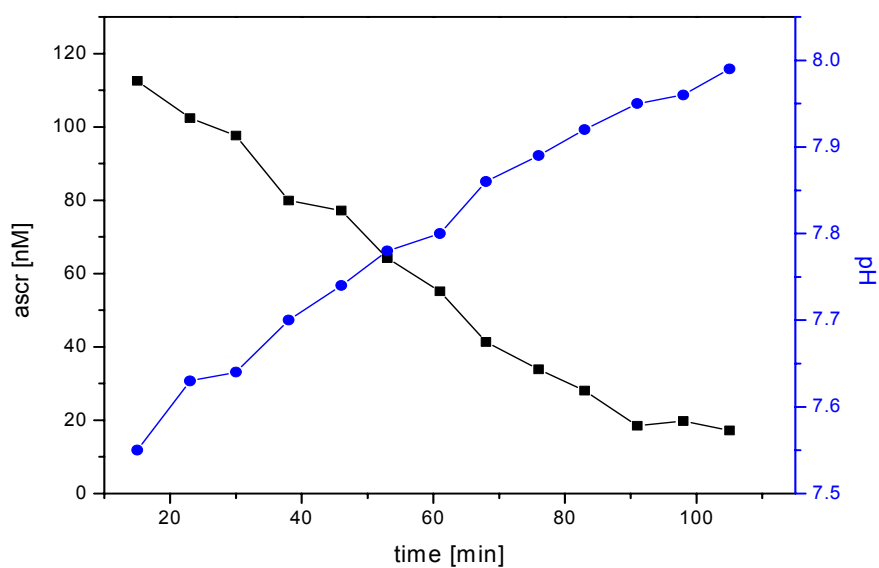
Figure 23   *Time dependent cross correlations between ascorbyl radical concentrations (—■—) and pH (—●—) 7.64 of plasma under laboratory conditions.*

3.1.5   Primary Parameter: ascorbic acid concentrations in blood plasma

The results presented in the previous section **3.1.3** provide some information on reactions of ascorbyl radicals in healthy subjects. Is it possible to observe correlations between ascorbyl
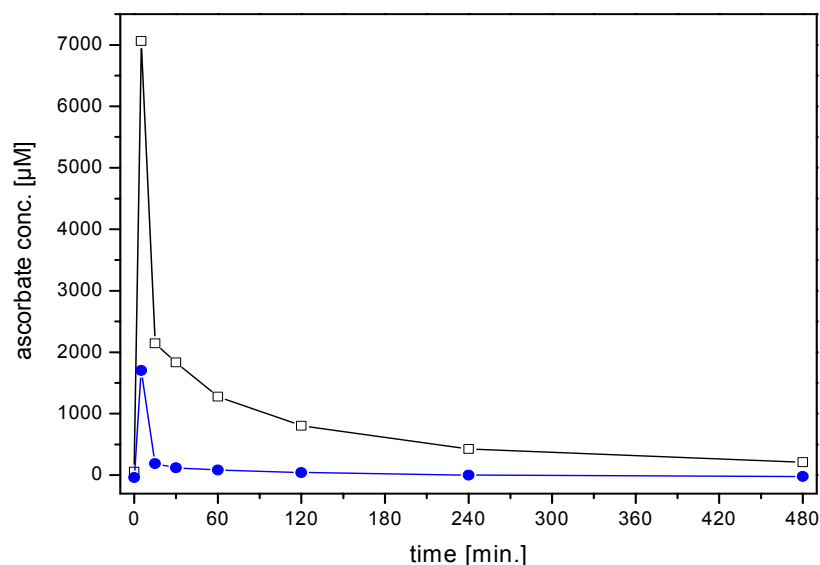
Figure 24 *Ascorbate levels in blood plasma of 6 healthy subjects after infusion of 750 mg* (—●—) *and 7.5 g* (—□—) *vitamin C on day 1 and day 23*. Ave. stdev. 7.5 g = ±793, ave. stdev. 750 mg = ±230. Courtesy: Silke Mrosek.

radical and ascorbate concentrations? To prove this, ascorbyl radical concentrations during a time course of 8 hours were compared with the ascorbate plasma concentration as determined at the University of Hohenheim[*].

The ascorbate and ascorbyl radical concentrations in plasma of healthy subjects after vitamin C infusion followed similar non-linear pathways (**Figure 24**). However, vitamin C infusion induced a sharp increase in ascorbate plasma concentration initially. This was followed by a continuous decrease after 5 minutes. Subsequently, ascorbate plasma concentrations relatively stabilized after 2 hours of vitamin C infusion. Nevertheless, even after 8 hours ascorbate levels in plasma had not completely disappeared.

In order to study the correlation between ascorbate concentrations and ascorbyl radical formation in plasma after vitamin C infusion, their concentrations were compared at different doses of vitamin C over the course of 8 hours (**Figures 25 and 27**). Data were replotted for cross correlation between ascorbate and the radical concentrations before and after 1, 2, 4 and 8 hours of vitamin C infusion from healthy subjects (**Figures 26** and **28**).
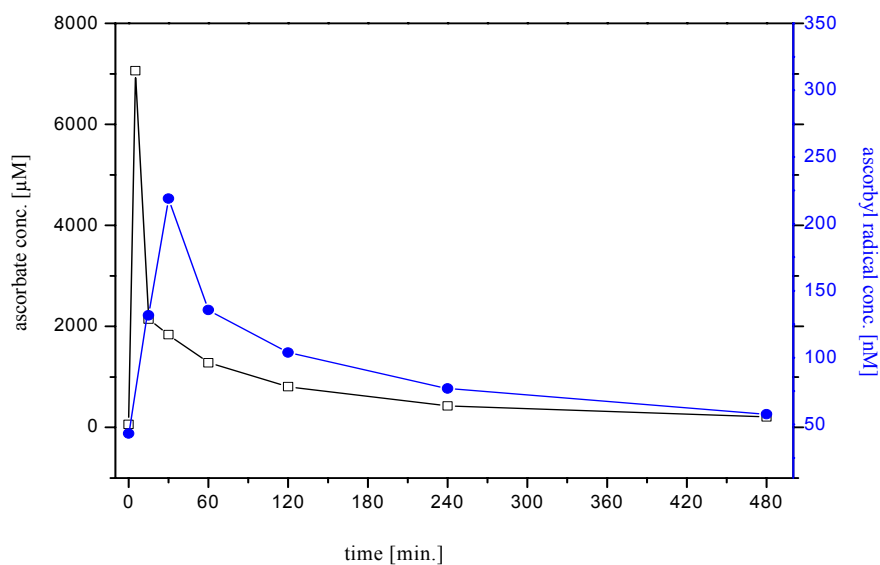
Figure 25 *Ascorbate* (–□–) *and ascorbyl radical* (–●–) *concentrations in blood plasma of 6 healthy volunteers after the infusion of 7.5 g vitamin C on day 1 and day 23.*
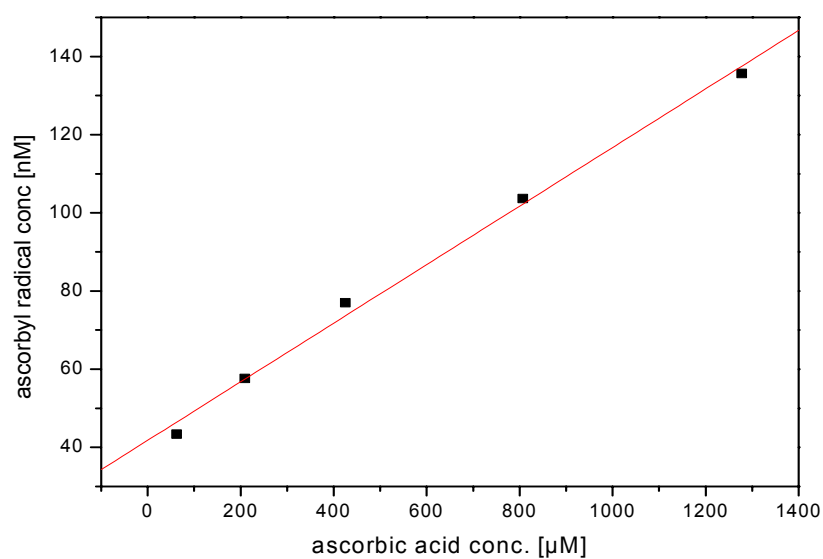
Figure 26 *Correlation between ascorbate levels versus ascorbyl radical concentrations in blood plasma before 7.5 g vitamin C infusion and 1, 2, 3, 4, hours* (–■–) *after infusion of healthy volunteers. Slope B = 0.07 ± 0.003, R = 0.99.*

* *Institut für Biologische Chemie und Ernährungswissenschaft an der Universität Hohenheim. Silke Mrosek et al.*
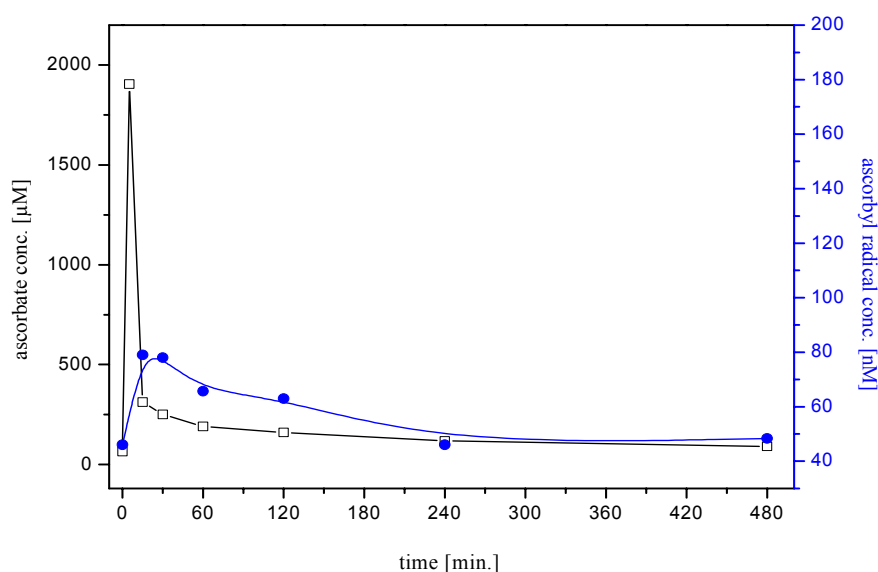
Figure 27 *Ascorbate* (–□–) *and ascorbyl radical* (–●–) *concentrations in blood plasma of 6 healthy volunteers after infusion of 750 m g vitamin C on day 1 and day 23.*
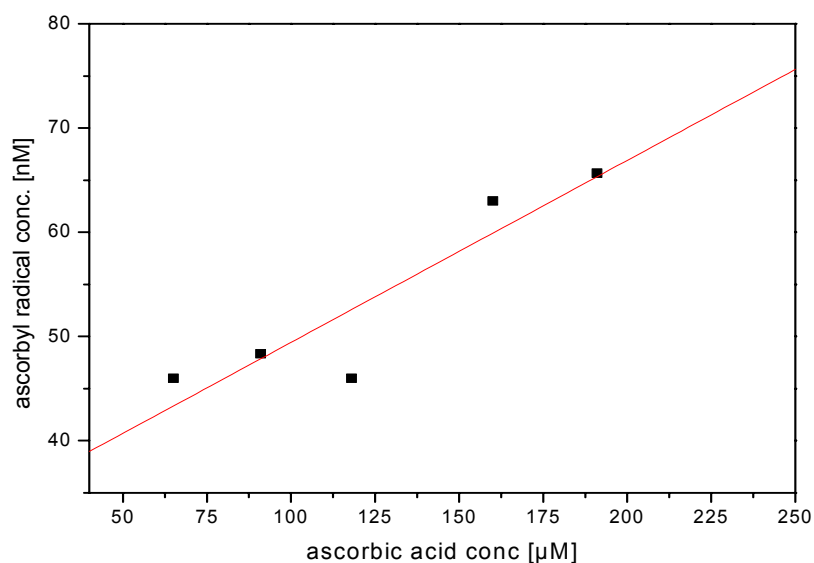
Figure 28 *Ascorbate concentrations versus ascorbyl radical concentrations in blood plasma before 750 mg vitamin C infusion and 1, 2, 3, 4, hours* (–■–) *after infusion of healthy volunteers. Slope B = 0.17 ± 0.04, R = 0.92.*

The results show that positive linear correlations exist between ascorbate and ascorbyl free radicals for both the groups supplemented with vitamin C. Healthy subjects supplemented with 7.5 g vitamin C revealed a correlation slope of B = 0.07 (**Figure 26**)**,** whereas 750 mg

vitamin C supplements showed slopes of B = 0.17 (**Figure 28**). Apparently, good linear correlations between the plasma ascorbate and ascorbyl radical concentrations after vitamin C infusion in healthy subjects reveal the relationship between ascorbate oxidation and the formation of ascorbyl radicals in the plasma.

3.1.6 Biochemical correlations between secondary parameters in blood plasma and urine from healthy subjects with ascorbyl radical concentrations

To review the controversies on the effects of pro-oxidative damage due to heavy doses of vitamin C exceeding the recommended dietary allowance in humans, the results obtained from the biochemical secondary parameters for oxidative stress measured at the university of Hohenheim* were compared with ascorbyl radical concentrations. They include vitamin E and malondialdehyde (MDA) levels in plasma and 8-oxoguanine, oxalate and calcium levels in urine. Secondary parameters were correlated with ascorbyl radical concentrations before and at days 1 and 6 after vitamin C infusion.
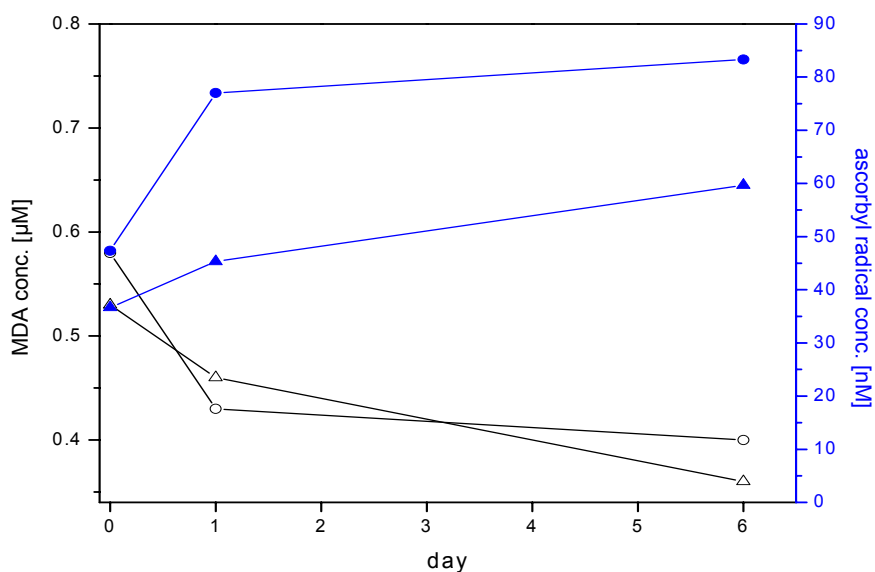
Figure 29   *Correlation between the ascorbyl radical (−▲−), (−●−) and MDA (−△−), (−○−) concentrations before and after 750 mg, 7.5 g vitamin C infusion on days 1 and 6.*

* Biologische Chemie und Ernährungswissenschaft der Universität Hohenheim. Silke Mrosek et al.

One of the widely used methods to determine lipid peroxidation during oxidative stress is the thiobarbituric acid-reactive substances (TBARS) assay. Production of reactive oxygen species on the onset of oxidative stress can directly attack lipids and proteins in the biological membrane at the local site of generation and cause their dysfunction. MDA is the degradation product of the major chain reactions resulting from the oxidation of polyunsaturated fatty acids and thus serves as a marker of oxidative stress.
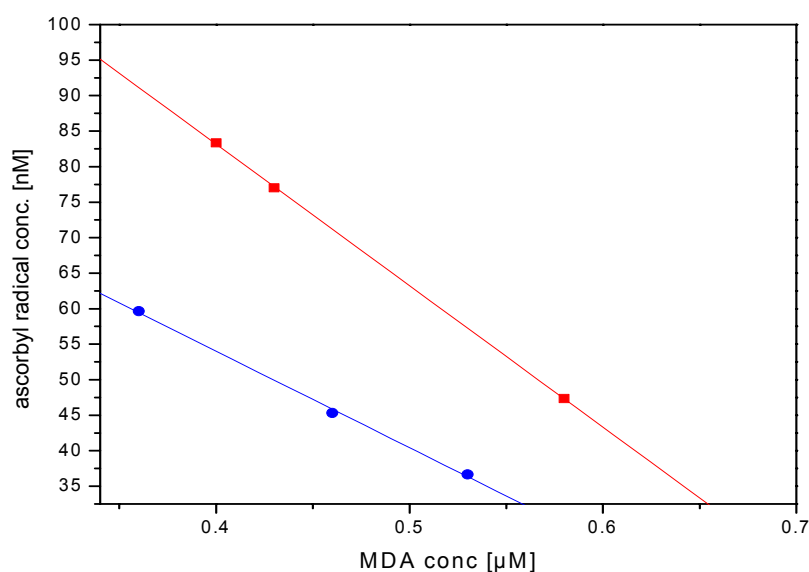
Figure 30   *Inverse linear correlations between ascorbyl radical and MDA concentrations before and after 750 mg (—●—), 7.5 g (—■—) vitamin C infusion on days 1 and 6.*

Malondialdehyde (MDA) levels in blood plasma were observed at days 1 and day 6 after vitamin C infusion (**Figure 29**). A systemic decrease in MDA concentration was seen in relation to ascorbyl radical concentrations after 6 days of vitamin C supplementation. Pro-oxidative properties, as a consequence of ascorbyl radicals in plasma, would have favoured an increase in MDA levels. However, MDA concentrations decreased during 6 days of vitamin C infusion. Indeed, a linear inverse correlation between ascorbyl radical and MDA concentrations could be observed as shown in **Figure 30**, with a steeper slope for subjects with higher doses of vitamin C. This may reaffirm the antioxidant efficacy of ascorbic acid by inhibiting lipid peroxidation.
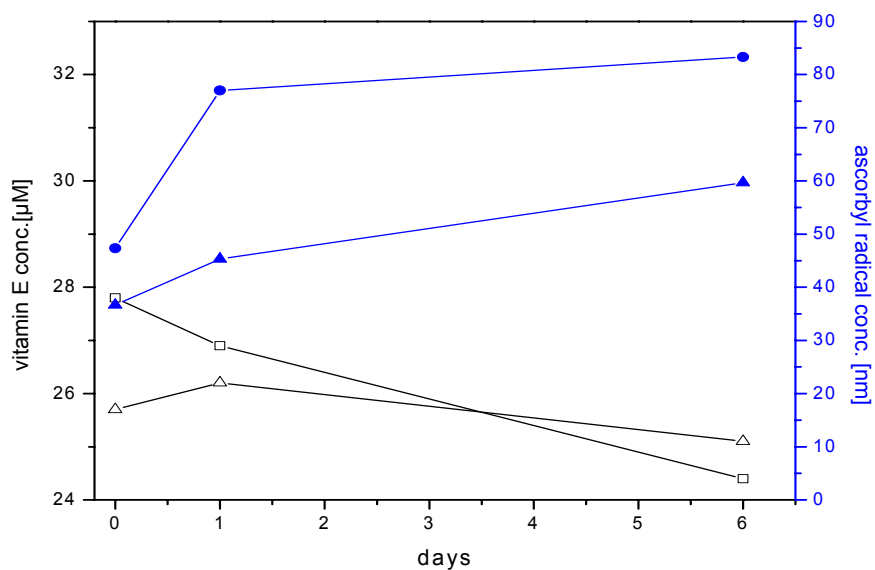
Figure 31   *Relationship between ascorbyl radical formation* (−▲−), (−●−) *and a marginal decrease in vitamin E concentration* (−△−), (−○−) *in plasma after 750 mg, 7.5 g vitamin C infusion.*

Comparison of the ascorbyl radical concentration with vitamin E levels showed that in spite of significant increase of the ascorbyl radical concentrations, vitamin E levels decreased only marginally in the course of 6 days of vitamin C infusion (**Figure 31**).

No significant effects on oxidative stress were observed with other parameters such as 8-oxoguanine, calcium and oxalate (Figures not shown) as a result of high ascorbyl concentrations in plasma after vitamin C supplementation. Consequently, from this study it can be concluded that increasing vitamin C intake in the form of an infusion exhibits no short-term effects of pro-oxidative stress in healthy subjects.

In summary, studies on healthy subjects showed significant increase in ascorbyl radical concentrations immediately after infusion, which decreased steadily after reaching a maximum. This time-related pattern in the increase of ESR signal intensity followed by its decrease corresponds to the two-step oxidation of ascorbate in which single electrons are transferred. The oxidation pattern of ascorbate oxidation showed similarities to various reactions of ascorbate in this thesis. In healthy subjects there are many possibilities that result in an increase in ascorbyl radical concentrations. They include radical intermediates resulting from mitochondrial metabolism, auto-oxidation of haemoglobin and free metal-catalyzed reactions. In normal subjects, ascorbate concentration in plasma is determined by dietary

intake, renal loss, and absorption by tissues and redox processes. Thus, the generation of ascorbyl radicals may be taken to be equal to the total rate of reactions of free radicals with ascorbate during regular metabolic processes and/or through periods of oxidative stress. Therefore, the oxidation of ascorbate to ascorbyl radical in healthy subjects may allow to indirectly estimate the rate of oxidative transformations.

Correlations of free radical oxidation of ascorbate with secondary parameters of oxidative stress determined at the University of Hohenheim did not show prooxidative properties of heavy doses vitamin C. Indeed, MDA levels decreased with an increase in ascorbyl radical concentrations and ascorbate oxidation did not influence vitamin E, 8-oxoguanine and oxalate levels.

3.2   Biochemical correlations of oxidative stress parameters from patients undergoing an aorta-coronary bypass operation after heavy doses vitamin C therapy

The main aim of this project was to study the efficacy of heavy doses of vitamin C during periods of oxidative stress as a consequence of reperfusion and reoxygenation of patients undergoing an aorta-coronary bypass operation (CABO).

Oxidative stress plays a central role in the pathogenesis of ischemic heart diseases and atherogenesis, for cancer and other chronic diseases in general, and it is also an important factor in aging processes. Therefore the role of antioxidants like vitamin C may indicate a correlation between ascorbyl radical concentrations and oxidative stress in the blood plasma of patients undergoing CABO. Moreover, pro-oxidative effects if arising due to very high vitamin C concentrations are to be studied. Also secondary parameters of oxidative stress in relation to vitamin C dosage for example ABTS for total antioxidant capacity, TBARS to determine MDA levels, sICAM and VCAM determined at the university of Hohenheim[*] are to be compared with the controversial pro-oxidative aspects of high ascorbyl radical concentrations.

3.2.1   Classification of patients in treatment groups

Patients given ascorbic acid intravenously were classified into three groups. Group one was treated with placebo, patient group two given 7.5 g ascorbic acid as bolus and continuous infusion of 22.5 g for 24 h and the third group with 15 g as bolus and continuous infusion of 30 g ascorbic acid for 24 h (**Table 2**).

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* Vitamin C, TAS und TBARS: Marina Langer, Universität Hohenheim, Institut für Biologische Chemie und Ernährungswissenschaft

* VCAM und ICAM: Beate Schlegel, Universität Hohenheim, Institut für Biologische Chemie und Ernährungswissenschaft

Group	group 1	group 2	Group 3	Total
Patients	17	20	20	57

Table 2 *Patients were divided into three groups: Group 1; placebo, group 2; 7.5 + 22.5 g and group 3; 15 + 30 g treated with vitamin C infusion.*

Ascorbyl radical concentrations were compared with other parameters for oxidative stress, such as total antioxidant capacity, MDA, sICAM and sVCAM assays. Ascorbyl radical concentrations were measured at the time points given below:

Time points B

1. *Morning before surgery*
2. *Immediately before stenosis*
3. *1 minute after reperfusion*
4. *5 minutes after reperfusion*
5. *20 minutes after reperfusion*
6. *Immediately after surgery*
7. *2 hours after surgery*
8. *4 hours after surgery*
9. *1 day after surgery*
10. *3 days after surgery*

3.2.2 Determination of ascorbyl radical concentrations using ESR spectroscopy and its relation to ascorbic acid concentrations

Aorta-coronary bypass can induce injury as a result of inflammatory processes induced by reactive oxygen species and tissue damage can also be a product of reperfusion or reoxygenation injury. In order to study ascorbate oxidation during surgery, a correlation between the ascorbyl radical concentration and ROS formed during periods of oxidative stress was established. Since ascorbic acid is considered a natural indicator of oxidative stress, redox reaction of ascorbate is studied in patients undergoing CABO. One way to achieve this is by comparing time-dependent changes in ascorbyl radical concentrations with ascorbate concentrations and other parameters of oxidative stress. In addition, pro-oxidative aspects of

heavy doses of ascorbate were studied with respect to other biochemical parameters of oxidative stress.
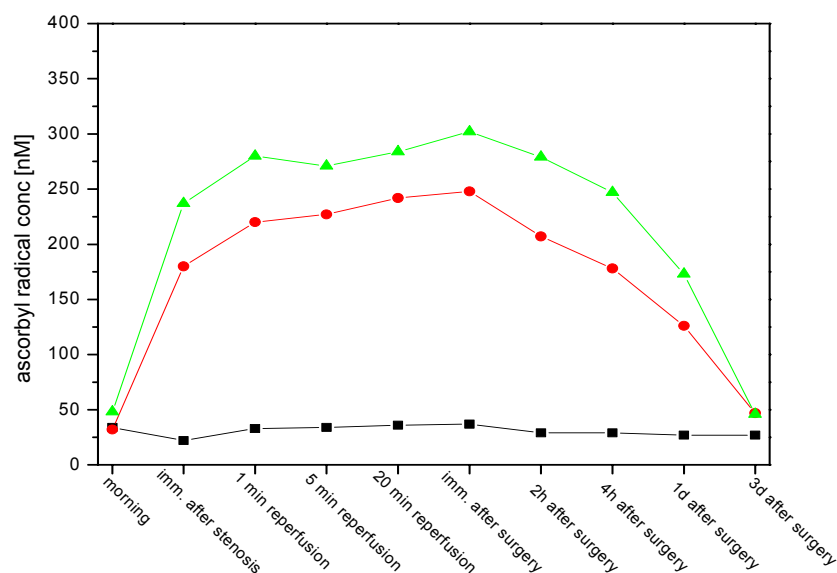
Figure 32 *Ascorbyl radical concentrations after placebo (–■–), 22.5 + 7.5 g (–●–) and 30 +15 g (–▲–) vitamin C infusion of patients during CABO and reperfusion. Placebo ave. stdev. = ±8, 30 g ave. stdev. = ±49, 45g, ave. stdev. = ± 67.*

Ascorbyl radicals that are formed by ascorbate oxidation were determined using ESR spectroscopy as seen in the previous chapter. In patients after vitamin C infusion, plasma ascorbyl radical concentrations increased significantly compared to placebo patients as seen in **Figure 32**. Ascorbyl radical concentrations remained relatively stable during reperfusion and surgery. However, a marginal increase in radical concentrations was observed particularly during reperfusion and immediately after surgery. After 2 hours of surgery ascorbyl radical concentrations decreased steadily as seen in healthy subjects after vitamin C infusion. Consequently, does ascorbate follow similar kinetics as observed in ascorbyl radicals? As shown in **Figure 33**, ascorbate and ascorbyl radical concentrations exhibit similar dose-response curves after vitamin C infusion. A significant increase in ascorbate and ascorbyl radical concentrations was observed immediately after vitamin C infusion. Maximum ascorbate concentrations were seen immediately after reperfusion, which decreased steadily after
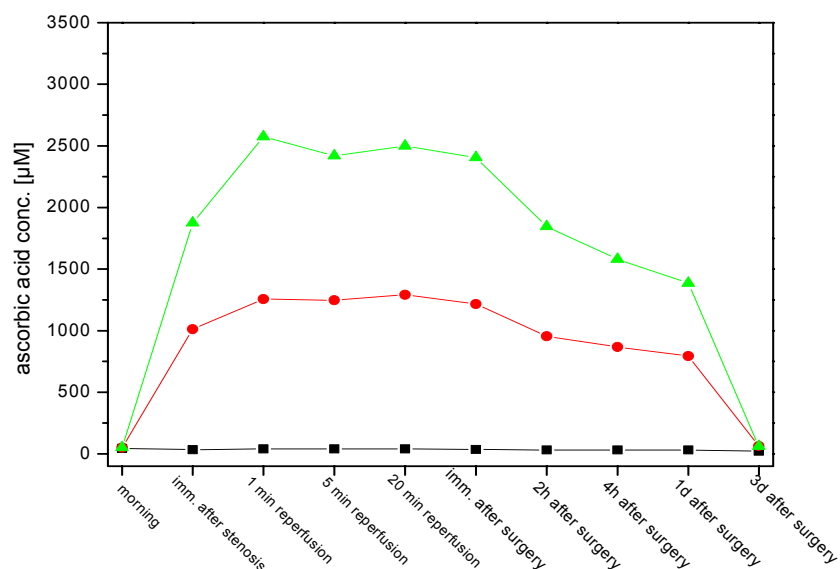
Figure 33 *Ascorbate levels in plasma from patients during CABO after placebo (–■–), 7.5 + 22.5 g(–●–), and 15 + 30 g(–▲–) vitamin C infusion before and after reperfusion and surgery. Placebo ave. stdev. = ± 9, 30 g ave. stdev. = ± 307, 45 g ave. stdev. = ± 670. Courtesy: Marina Langer.*
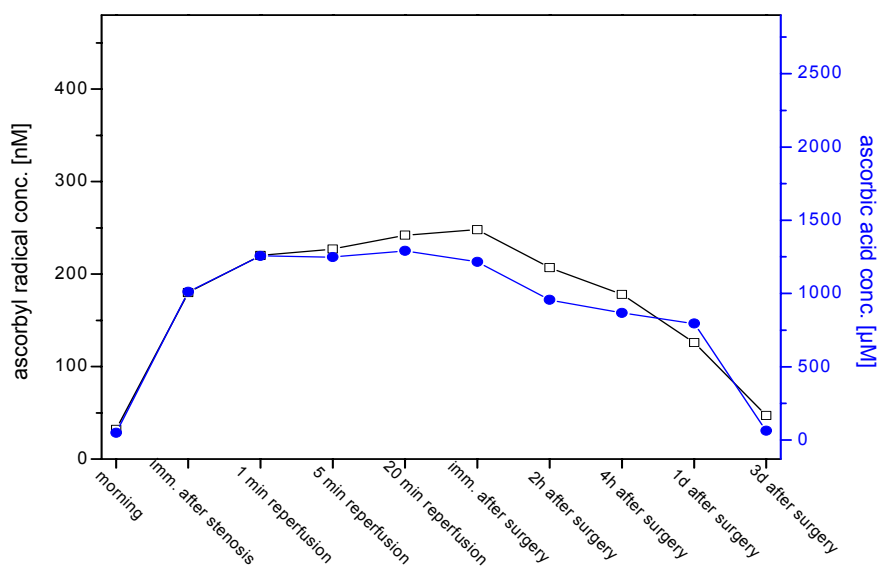
Figure 34   *Relationship between ascorbic acid (–●–) and ascorbyl radical concentrations (–□–) in blood plasma of patients undergoing CABO after 7.5 + 22.5 g vitamin C infusion.*

surgery. It is known that, under oxidative stress, the consumption of ascorbate can be high, and without regeneration, ascorbate would soon be depleted. Nevertheless, by comparing

ascorbate and ascorbyl radical concentrations, some changes in their levels were seen in relation to placebo patients and patients after vitamin C treatment (**Figures 34**, **35** and **36**).
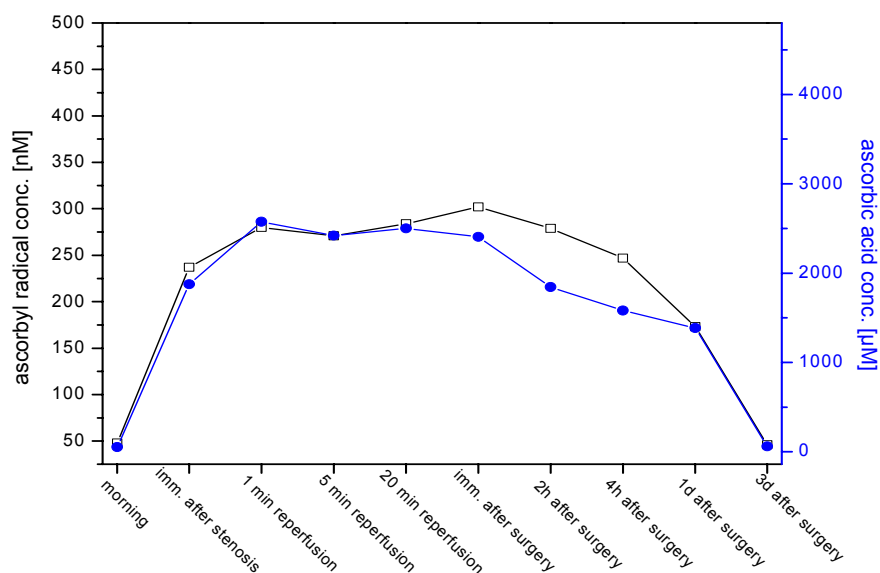
Figure 35 *Correlation between ascorbic acid levels (–●–) and ascorbyl radical concentrations (–□–) in blood plasma of patients undergoing CABO after 15 + 30 g vitamin C infusion.*
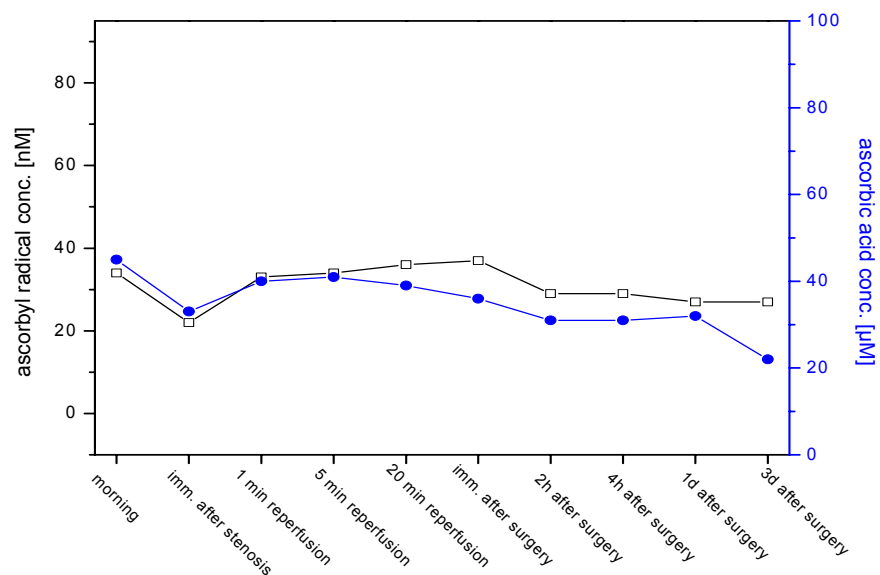
Figure 36 *Average ascorbic acid levels (–●–) in relation with ascorbyl radical concentrations (–□–) in blood plasma of placebo patients undergoing CABO during a course of 3 days.*

The changes in their plasma concentrations occurred predominately during reperfusion and immediately after surgery. Interestingly, both ascorbyl radical and ascorbate concentrations increased slightly during reperfusion. However, ascorbyl radical concentrations increased still further, whereas ascorbate levels fell marginally. The superimposed time-dependent curves of ascorbyl radical and ascorbate levels in plasma showed inverse correlations 20 minutes after reperfusion and immediately after surgery in all the examined patients with and without vitamin C treatment (**Figures 34**, **35** and **36**). However, concentrations of ascorbate and the ascorbyl radical differ by 4 orders of magnitude.

Nevertheless, the changes in ascorbyl radical concentrations were more prominent in patients after vitamin C treatment. What is the physiological relevance of ascorbate in patients after vitamin C treatment? Also, does the increase in radical concentration during reperfusion and surgery imply oxidative stress? To understand the mechanism of ascorbate oxidation it is necessary to gain some background information on the intra- and extracellular accumulation of ascorbate *in vivo*.
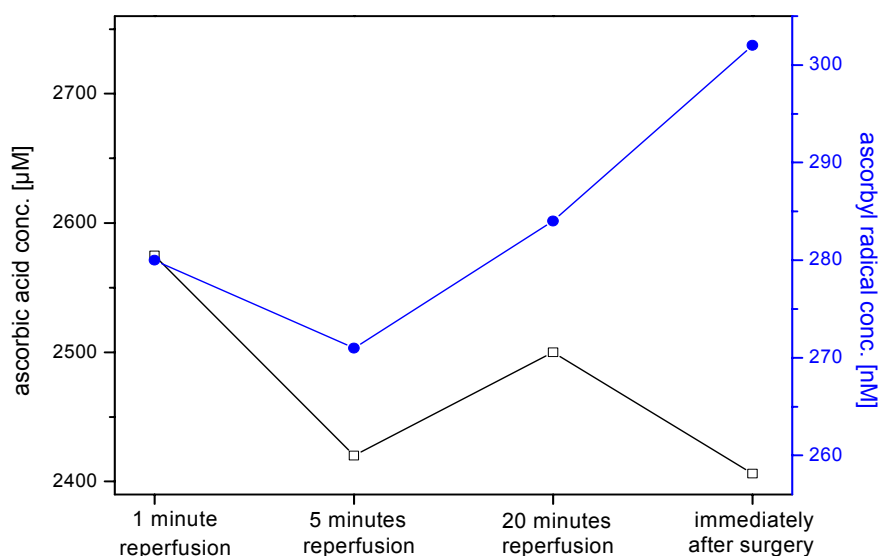
Figure 37 *Increase in ascorbic acid (–●–) and ascorbyl radical plasma concentrations (–□–) during 20 minutes reperfusion from patients undergoing CABO after 15 + 30 g vitamin C infusion. A further increase in ascorbyl radical concentration was seen in relation to ascorbic acid concentration.*

In several publications dealing with studies on ascorbate oxidation, ascorbate concentrations were followed both, in whole blood as well as white blood cells. It was shown that the blood cells contain ascorbic acid even when it is not present in plasma.

Symptoms of scurvy were apparent only when a total depletion in the cellular ascorbic acid level was observed and the cellular values are closely related with the total body ascorbic acid concentration. In these studies both ascorbic acid as well as dehydroascorbate concentrations were determined [Butler and Cushman 1940, Crandon et al 1940, Heineman 1938, Lowry et al 1946, Roe et al 1947, Roe and Kuether 1946].

Therefore, an increase in ascorbyl radical and ascorbate concentrations observed immediately after surgery may involve intracellular ascorbate released during periods of oxidative stress and subsequent oxidation of ascorbate to ascorbyl radicals. This increase in ascorbyl radical concentrations overlays the existing radical concentrations already present in plasma. This may result in an overall elevation in ascorbyl free radical concentrations after reperfusion and surgery. An approximately 2-fold increase in ascorbyl radical concentrations in plasma was detected, after vitamin C supplementation, during reperfusion that further increased after surgery (**Figures 37, 38,** and **39**). Maximum ascorbyl radical levels were observed immediately after surgery. These results may reveal that tissue cells saturated with ascorbate tend to release it into the blood plasma during reperfusion and surgery. This phenomenon may be a consequence of oxidative stress generated during reperfusion and surgery that may trigger the release of intracellular ascorbate in agreement with existing literature [Sylvia et al 1994].
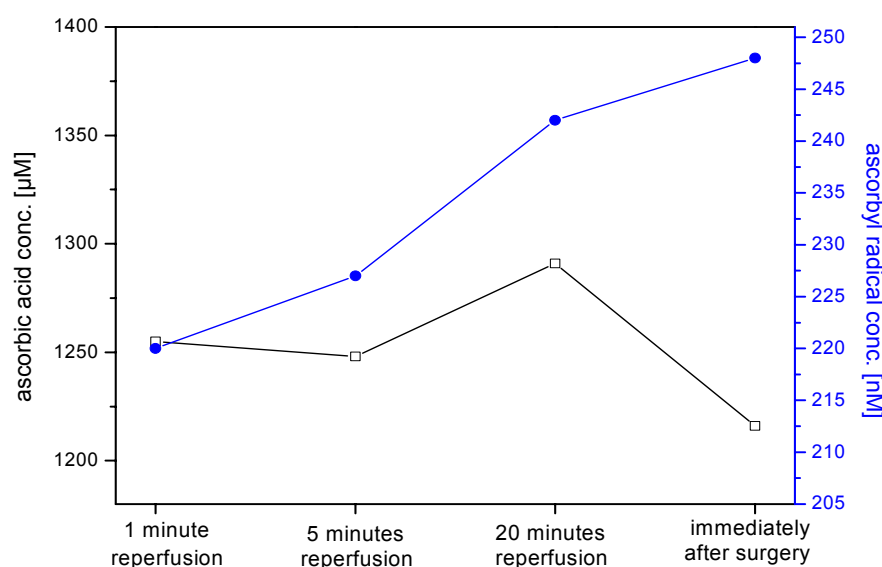
Figure 38 *An increase in ascorbic acid levels (–□–) and ascorbyl radical plasma concentrations (–●–) over a period of 20 minutes of reperfusion from patients undergoing CABO after 7.5 + 22.5 g vitamin C infusion. A marginal decrease in ascorbic acid concentrations was seen after reperfusion.*
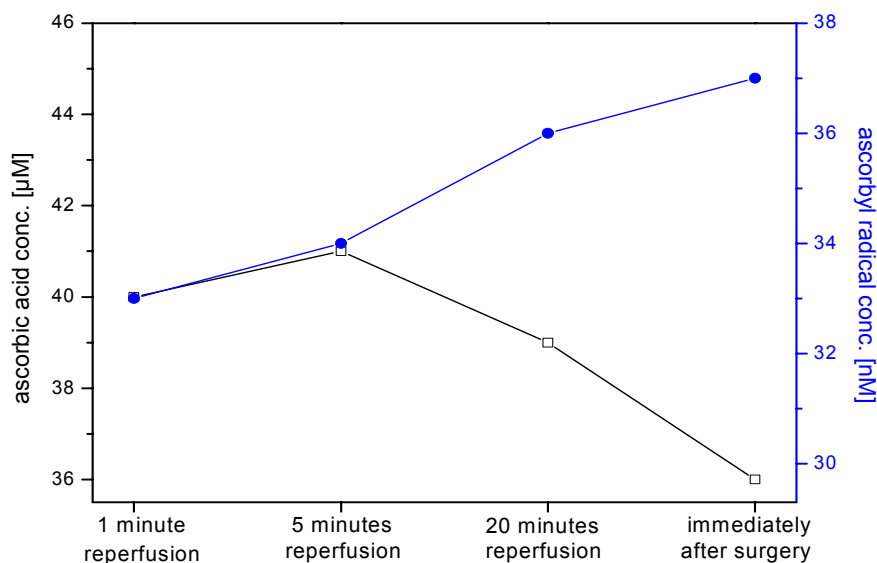
Figure 39 *An increase in ascorbyl radical plasma concentrations (–•–) was seen after reperfusion and surgery of placebo patients undergoing CABO. Ascorbic acid concentrations (–□–) decreased after 5 minutes reperfusion.*

Subsequently, ascorbate and ascorbyl radical plasma concentrations decreased steadily for 2 hours and baseline concentrations were observed after 3 days.

In conclusion, the evidence suggests that reperfusion and cardiac bypass surgery resulted in the release of free oxygen radicals. Ascorbyl radical concentrations before, during and after reperfusion and surgery were followed using ESR and compared with ascorbate concentrations in plasma. An increase in ascorbyl radical concentrations particularly after reperfusion and surgery may indicate the presence of free radical species, which were ultimately scavenged by vitamin C administered to patients in large doses. Although the amount of vitamin C administered was far above the recommended dietary allowance by RDA, evidence acquired using biochemical parameters of oxidative stress as mentioned in the previous chapter literally showed no adverse effects on healthy subjects. To prove these results, the efficacy of vitamin C in large doses for patients undergoing CABO is to be investigated by comparing the above data with other biochemical parameters for oxidative stress.

3.2.3 The relationship between ascorbyl free radical concentrations and secondary parameters of oxidative stress; TBARS, sICAM and sVCAM and total antioxidant capacity assays determined in Hohenheim

Polyunsaturated lipids are very susceptible to free-radical oxygen species. This process, referred to as lipid peroxidation, eventually yields several relatively stable decomposition products, including aldehydes that can then be measured in plasma as an indirect index of free-radical activity. Malondialdehyde, likely the most commonly measured index of oxidative stress in human studies, is only one of many aldehyde compounds produced by lipid peroxidation. Malondialdehyde is frequently measured in plasma by the thiobarbituric acid-reactive substances (TBARS) assay. Thiobarbituric acid reacts with malondialdehyde to produce a red stable adduct that was quantified using fluorescence spectroscopy in Hohenheim[*].
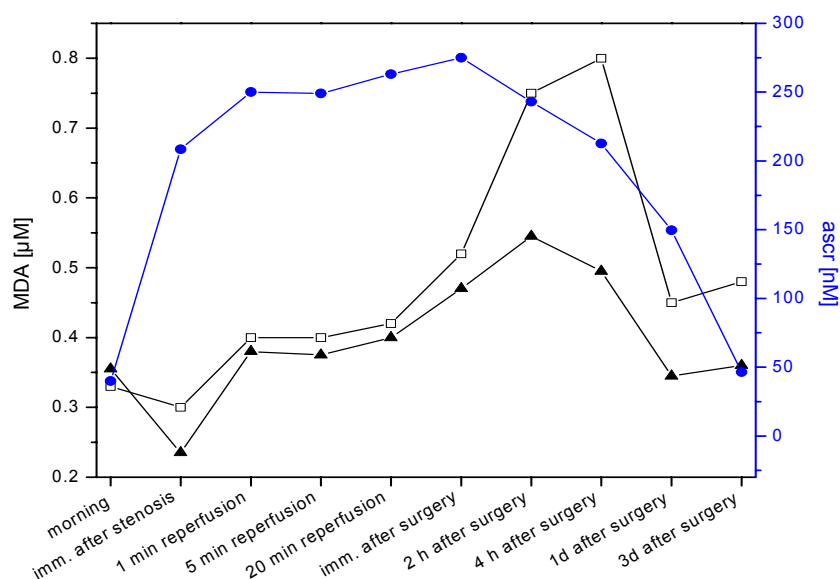
Figure 40 *MDA concentrations between the group of placebo patients (–□–) and patients after vitamin C infusion (–▲–) in relation to ascorbyl radical concentrations (–●–). Placebo average stdev = ± 0.16 and ± 0.11 for vitamin C traeated patients. Courtesy: Marina Langer.*

MDA and ascorbyl radical concentrations of patients after vitamin C infusion were averaged and compared with MDA concentrations of placebo patients undergoing CABO as shown in **Figure 40.** MDA concentrations of placebo patients compared to patients after vitamin C infusion were considerably higher.

Highest MDA concentrations were seen in placebo patients particularly 4 hours after surgery. Patients treated with 30g vitamin C showed lowest MDA concentrations during reperfusion and surgery (**Figure** not shown).

The above results reveal that patients treated with heavy doses of vitamin C show lower MDA concentrations. This may signify the vital role of vitamin C at higher concentrations, which may act as a consistent water-soluble antioxidant. Ascorbic acid is known to prevent the oxidation of LDL by scavenging ROS in aqueous medium. High ascorbate concentrations in plasma can strongly inhibit LDL oxidation by vascular endothelium cells. Controversial to a suggested vitamin C supplementation of about 75 to 500 mg/ day, much higher doses of ascorbic acid caused no pro-oxidative effects on patients after undergoing a coronary aorta bypass operation.

Inflammation due to oxidative stress is accompanied by an increase in cell adhesion molecules (ICAM and VCAM). There are several different structural groups of adhesion factors which have been identified on endothelial cells and which interact with receptors of leukocytes and platelets. Intracellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) are cell surface glycoproteins found on many cell types. ICAM-1 is inducible on cultured endothelial cell by inflammatory mediators as interleukin-1 (IL-1), tumour necrosis factor (TNF), interferon-γ (IFN-γ) and endotoxin. ICAM-1 can bind lymphocytes, monocytes and neutrophils to endothelium. Vascular cell adhesion molecule-1 (VCAM-1) is also induced by cytokines and binds selectively to lymphocytes and to some monocytes, but not to neutrophils *Bevilacqua et al 1994*.

Oxidative stress and expression of adhesion molecules, ICAM-1 and VCAM-1 are early features in the pathogenesis of atherosclerosis and other inflammatory diseases. Antioxidants have been suggested to have therapeutic potential in pathologies related to changes in cellular adhesion. Antioxidant, at micromolar concentrations (achievable in human/animal plasma following nutritional supplementation) down-regulates agonist-induced adhesion of leukocytes to endothelial cells. Although it has been consistently documented that antioxidants potently suppress inducible ICAM-1 as well as VCAM-1 expression, understanding of the molecular mechanisms involved is vague at best. At present, studies in humans have investigated the role of ascorbate in inhibiting cell-cell adhesion *Weber and Wolfgang 1996, Adam, Jessup, and Celermajer 1997*.

To study the correlation between ascorbyl radical concentrations and sVCAM/ sICAM levels in plasma after vitamin C infusion, their results were compared with placebo patients. It was found that sVCAM/ sICAM levels in plasma were higher in placebo patients as compared to
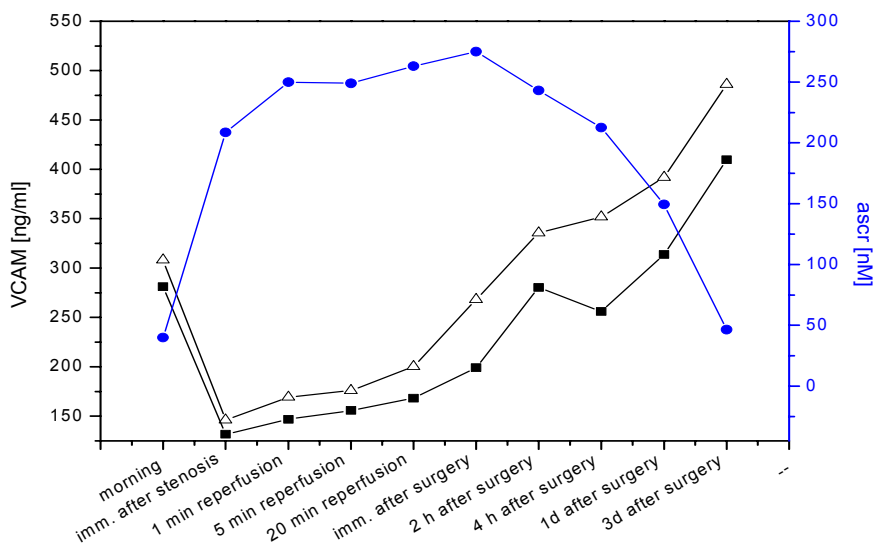
Figure 41   *Correlation between free radical ascorbate oxidation (–●–), sVCAM levels of patients after vitamin C infusion (–■–) and those of placebo patients (–△–). Courtesy: Beate Schlegel.*
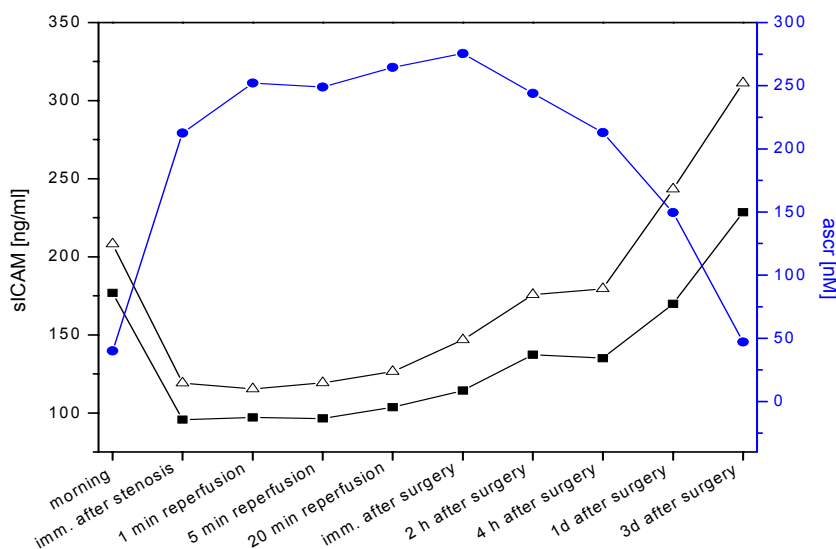
Figure 42   *Correlation between free radical ascorbate oxidation (–●–), sICAM levels of patients after vitamin C infusion (–■–) and those of placebo patients (–△–). Courtesy: Beate Schlegel.*

patients after vitamin C infusion (**Figures 41** and **42**).

The above results prove that patients after vitamin C dosage showed reduced sICAM and sVCAM levels during reperfusion and surgery. High ascorbyl radical concentrations did not cause an increase in sVCAM and sICAM levels as compared to placebo patients. Indeed, a marginal decrease in sICAM and sVCAM levels was seen after surgery.

An additional biochemical parameter for oxidative stress to study the pro-oxidative effects of vitamin C is the TAS assay. This method, which applies a simple direct antioxidant assay, based on the reduction of the ABTS.+ radical cation, and compares it with the myoglobin/ABTS.+ assay. The ABTS radical cation reacts quantitatively and instantaneously with several antioxidants, such as Trolox, ascorbic acid, uric acid, cysteine, glutathione and bilirubin. This procedure can provide an independent and simultaneous evaluation of the low molecular weight and protein antioxidants present in biological samples such as serum [Romay, Pascual and Lissi 1996].
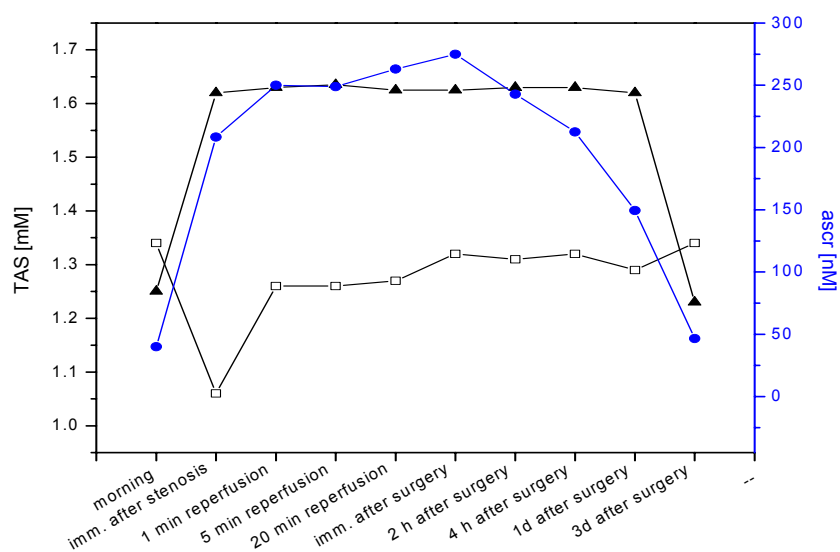
Figure 43   *Correlation between ascorbyl radical concentrations (–●–), total antioxidant capacity based on ABTS.+ radical cation levels of placebo patients (–□–) and the patients after vitamin C therapy (–▲–). Courtesy: Marina Langer.*

The total antioxidant capacity of patients after vitamin C infusion increased significantly in relation to those of placebo patients (**Figure 43**). The increase in total antioxidant status correlates well with ascorbyl radical concentrations in plasma during reperfusion and surgery.

3.2.4   Discussion on the effects of heavy doses of vitamin C on healthy subjects and patients undergoing bypass surgery

Vitamin C, an important water-soluble antioxidant in biological systems can significantly reduce or eliminate the adverse effects of reactive oxygen species and reactive nitrogen species on normal physiological functions in humans. The current dietary recommendation of vitamin C of about 60 - 100 mg per day based on threshold urinary excretion was initially applied for the prevention of scurvy. Nevertheless, antioxidant activity of vitamin C and its importance as cofactor in collagen, carnitine and neurotransmitter metabolism is not completely explored. Since most animals synthesize their own vitamin C and their dosage increases significantly during stress situations, new dietary allowance of vitamin C should be considered. Much higher doses might be appropriate in preventing vitamin C deficiency with a margin of safety, backed with biochemical parameters to eliminate controversies on pro-oxidative effects. Therefore, further studies were carried out by intravenous supplementation of heavy doses of vitamin C on healthy subjects and patients undergoing CABO.

Healthy volunteers were alternatively intravenously supplemented with 750 mg and 7.5 g vitamin C. The changes of ascorbyl radicals reveal the complex chemistry of free radical ascorbate oxidation in healthy subjects. The non-linear pathways of ascorbate and ascorbyl radical concentrations showed a systemic increase followed by a decrease that stabilized after 8 hours. Good linear correlations observed between ascorbate and ascorbyl radical concentrations confirm ascorbate oxidation. Moreover, the stability of ascorbyl radicals in plasma from healthy subjects at different concentrations was studied at normal laboratory conditions. Interestingly, higher ascorbyl radical plasma concentrations tend to show shorter half-lives. The redox processes responsible for this non-linear kinetics were influenced by pH changes in plasma containing EDTA as chelator. Also, metal mediated oxidation of ascorbate occurred after thawing of plasma probes caused by the release of free metal ions. An incomplete chelation of metal ions by EDTA resulted in an additional increase in ascorbate oxidation.

Alternatively, dose response curves of ascorbyl radicals in plasma *in vivo* revealed longer half-lives than *in vitro* experiments. This may be a consequence of ascorbate oxidation during regular metabolic processes and the reduction of dehydroascorbate. These processes show ascorbate as an efficient recycling antioxidant during oxidative stress. Correlations of ascorbyl radical concentrations with secondary parameters for oxidative stress determined at the University of Hohenheim revealed no significant changes before and after vitamin C treatment. Indeed MDA concentrations decreased after vitamin C supplementation supporting the relevance of vitamin C in diet as a water-soluble antioxidant.

Subsequently, the results obtained from the vitamin C treatment of healthy subjects enabled further investigations on oxidative stress with patients undergoing CABO. Bypass is an indispensable ingredient of cardiac surgery, but it can also inflict damage to several tissues and some patients can develop organ malfunction. It is believed to be activated by systemic inflammatory response to CABO, induced by the exposure of blood elements to nonphysiological surfaces [Morse et al 1998]. Furthermore, heart damage could also be associated with myocardial ischemia and reperfusion as a result of cross clamping which is clinically expressed as arrhythmia or "myocardial stunning", a depressed contractile function of major importance in the early postoperative period [Morse et al 1998, Bolli 1990, Opie 1989]. Therefore, ROS may play a significant role in these processes. An increase of various markers of oxidative stress [Lazzarino G et al 1994, Davies 1993, Ferrari et al 1990] has been demonstrated to occur during CABO.

Generation of oxygen free radicals could be the result of the activation of neutrophils occurring in response to an inflammatory reaction[Morse et al 1998]. Moreover, it is known that ROS generation takes place during myocardial ischemia and reperfusion in various experimental models[Lazzarino G et al 1994] and in human heart. ROS could therefore be responsible for bypass-induced damages or impairment of myocardial recovery[Curello et al 1995, Vergely 1998].

Patients undergoing coronary bypass operation (CABO) were treated with heavy doses vitamin C. Ascorbyl radical concentrations formed during reperfusion and surgery were correlated with other biochemical parameters of oxidative stress. Patients were classified into 3 groups: placebo, vitamin C treated patients with 7.5 g bolus and 22.5 g continuous-infusion and 22.5 g bolus and 30 g continuous-infusion. Dose-dependent changes in the ascorbate and ascorbyl radical levels in plasma were investigated as primary parameters of oxidative stress.

Ascorbyl radical and ascorbate concentrations before and after vitamin C infusion showed rather similar time courses. However after reperfusion and surgery, the increase in ascorbyl radical concentration was accompanied by a decrease in ascorbate concentration. This may prove the relationship between oxygen free radical formation and ascorbate oxidation during periods of oxidative stress

Secondary parameters for oxidative stress, i.e., MDA, sICAM and sVCAM and TAS assays were determined at the University of Hohenheim and were correlated with ascorbyl radical concentration after heavy doses of vitamin C infusion. These secondary parameters showed no significant pro-oxidative effects during reperfusion and surgery for treated patients in relation to placebo patients. Indeed, MDA, sICAM and sVCAM levels were lower than those of placebo patients after vitamin C treatment.

3.3   Chemistry and detection of ascorbyl free radicals and spin trapping techniques *in vitro* as markers of oxidative stress.

The dynamic chemistry of ascorbate makes it very interesting to reveal ascorbate oxidation and its oxidation products in the form of ascorbyl free radicals using ESR. Problems arising by the determination of ascorbyl free radical changes in varing concentrations and kinetics in non-physiological or cellular environment were to be studied. The indispensable function of ascorbic acid in its anti-oxidant defence mechanism lies on the endiol functional group on the lactone ring. Ascorbic acid has two ionisable hydroxyl groups ($AscH_2$) with pK_1 of 4.25, ionized at position 3, and at position 4 with a pK_2 of 11.8. Ascorbate easily donates a hydrogen atom to an oxidising radical to produce a resonance-stabilised tricarbonyl ascorbate radical ($Ascr^{\bullet}$) with a pK_a of -0.86; thus, it is not protonated at physiogical environment Buettner and Schafer .

Redox inter-conversions between ascorbate, ascorbyl radicals and dehydroascorbate *in vitro* were studied to monitor time-dependent changes in ascorbyl radical concentrations. Concentration dependent ascorbate oxidations can cause some problems in assessing ascorbyl radical concentrations. Fenton's reagent and xanthine-xanthine oxidase enzyme complex were used as source of oxygen centered radicals in experiments for ascorbate oxidation. Since the formation of ascorbyl free radical vastly depends on pH and ascorbate concentrations, redox behaviour was investigated under these conditions. Model systems with cell cultures were investigated, in the presence of ascorbate, under hypoxia, anoxia and reoxygenation and changes in ascorbyl radical intensities were followed up using ESR spectroscopy.

3.3.1   Redox reactions of ascorbate as a function of pH

The instability of the oxidized form of ascorbic acid is responsible for the variation in antioxidant potency and for the complex behaviour of ascorbate – ascorbyl radical and dehydroascorbate interaction *in vivo* and *in vitro*. To study the complex kinetics of ascorbate oxidation in biological systems, additional information are to be gathered in more simple systems and compared under the same conditions.

In this present work, ascorbate oxidation using hydrogen peroxide/ferrous ammonium sulphate as source of oxygen free radicals was studied as a function of pH. Buffers were prepared according to their pH ranges, with sodium phosphate-citrate buffer (pH 3.6 to 5.4), sodium phosphate buffer (pH 5.8 to 8.0) and borax buffer (pH 8 to 10). To determine ascorbyl radical concentrations, ascorbate was oxidized with 6 mM Fenton's reagent at different pH values and measured by ESR spectroscopy (**Figure 44**).
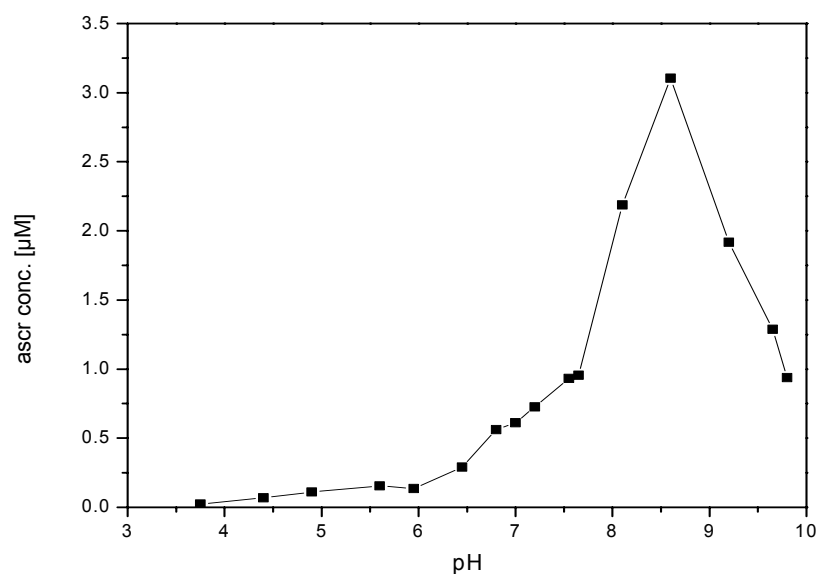
Figure 44 *Degree of free radical ascorbate oxidation as a function of pH. 6 mM of ascorbate was oxidized with 0.6 mM Fenton's reagent. pH range: 3.6 – 5.4 sodium phosphate-citrate buffer, 5.8 – 8.0 sodium phosphate buffer and 8.0 – 10 borax buffer. Stdev., min =± 0.02 at pH 3.7, max =± 0.4 at pH 9.8.*

The results shown in **Figure 44** reveal the dependence of ascorbyl radicals in the pH range from 3 to 10. At low pH, minimal ascorbyl radical intensity was seen which remained unaffected till pH 6. Between pH 6 and 8, radical concentrations increased moderately, probably as a consequence of ionization at position 3 on the hydroxyl group. Above the pH range of 8, a significant increase in ascorbyl radical intensity was seen, which reached a maximum at pH 8.8. Therefore, these results suggest that pH can greatly influence ascorbate oxidation caused by the step-wise ionization of the en-diol group on the lactone ring. At pH values above 8, the dianionic form of ascorbic acid may outweigh the mono anionic form shifting its equilibrium to dehydroascorbate. A rapid decrease in ascorbyl radical concentrations was observed at pH above 9. However, a previous paper showed [Wang et al 2002] a

decrease in ascorbyl radical levels only above pH 10. This decrease in ascorbyl radical concentration may be the result from the choice of the buffer used for this study.

Investigations on the dependence of pH on ascorbyl radical intensity are of great importance, since blood withdrawn in monovettes containing EDTA influenced plasma pH. Therefore, ascorbate oxidation must be carefully scrutinized by verifying the pH subsequently after measurments to eliminate experimental errors in studies on oxidative stress *in vivo* and *in vitro*.

3.3.2 Ascorbate oxidation with hypoxanthine/ xanthine-xanthine oxidase (XOD) *in vitro* as source of ROS

Xanthine oxidase is a highly versatile enzyme that is widely distributed among species and within various tissues of mammals. XOD plays an important role in the catabolism of purines. It belongs to the group of enzymes called molybdenium iron-sulphur flavin hydroxylases. XOD catalyses the hydroxylation of purines such as hypoxanthine, which is oxidized to xanthine with the release of superoxide anion as intermediates. Eventually, xanthine is further oxidized by XOD forming uric acid.

$$\text{Hypoxanthine} + O_2 + H_2O \xrightarrow{\text{XOD}} \text{Xanthine} + H_2O_2 + \cdot O_2$$

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{XOD}} \text{Uric acid} + H_2O_2 + \cdot O_2$$

Xanthine oxidase is a major source of oxygen free radicals. The enzyme catalyzes the reduction of oxygen to superoxide radical anion and hydrogen peroxide. This enzyme may play an important role in oxidative injury and/ or ischemia/ reoxygenation injury. Under hypoxia the depletion of the cell ATP results in elevated levels of AMP, which is further degraded to adenosine, inosine and hypoxanthine. Simultaneously xanthine dehydrogenase is decomposed to xanthine oxidase by a protease activated by excess cytosolic calcium levels during ischemia. The generation of superoxide radical anion as intermediates in xanthine

oxidase/ xanthine may be used as a source for *in vitro and in vivo* generation of free radicals. Incubation of hypoxanthine in xanthine oxidase PBS medium at physiological pH in labortory conditions led to a sequential depletion of hypoxanthine along with formation of uric acid. Time-dependent changes in hypoxanthine oxidation and uric acid generation were detected by UV spectroscopy at wavelength of 252 nm and 283 nm respectively as seen in **Figure 45**. By adding 0.312 mM ascorbate in hypoxanthine/xanthine oxidase systems, it was possible to follow ascorbate oxidation, as a result of the formation of superoxide radical anion intermediates.
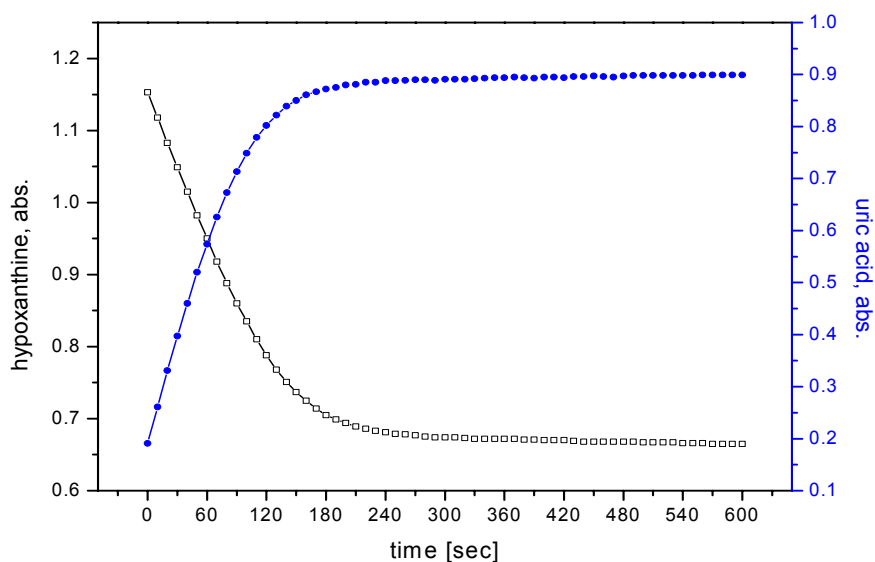
Figure 45 *Xanthine oxidase as a source of superoxide radical anion showing the kinetics of 10 mM hypoxanthine oxidation at λ_{max}=252 nm (−□−) and the formation of uric acid at λ_{max}=283 nm (−●−) in PBS at pH 7.4.*

Results in **Figures 46 and 47** demonstrate *in vitro* oxidation of ascorbate leading to the formation of ascorbyl radicals. Non-linear time-course of ascorbyl radical formation and its decay may reveal some information about oxidative stress in biological systems. In this study, 0.312 M ascorbate was incubated in 10 mM hypoxanthine substrate and the enzyme xanthine oxidase was used as free radical generating system.
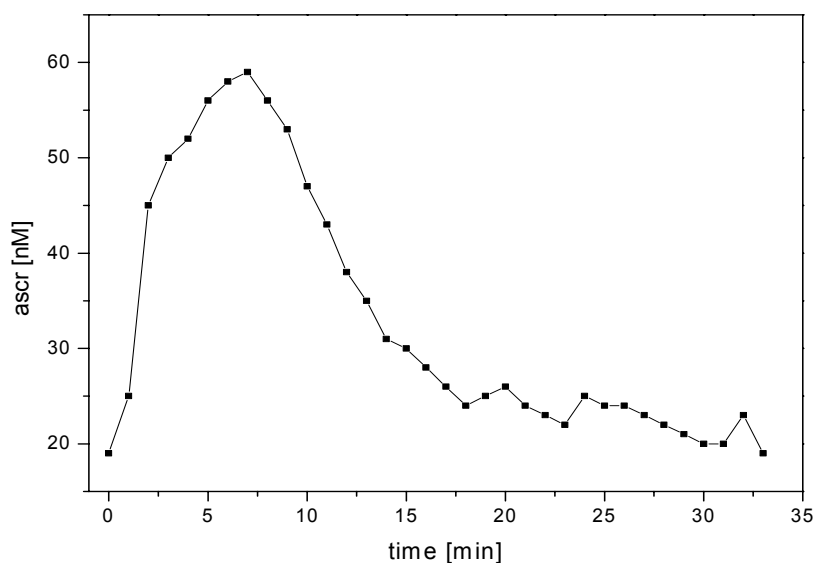
Figure 46 *Oxidation of 1mM ascorbate with hypoxanthine/ xanthine oxidase system in PBS at pH 7.4 after the removal of metal ions.*

Time-dependent studies showed an increase in ascorbyl radical concentrations within the first few minutes, which decreased steadily after that (**Figure 46**). This non-linear pathway suggests the oxidation of ascorbate following a two-step reaction that yields an ascorbyl radical at the first step and a dehydroascorbic acid in the second step. To study the changes in ascorbyl radical concentrations, hypoxanthine breakdown and uric acid formation, diagrams were superimposed as seen in **Figures 47** and **48**. These reactions show a poor linear correlation between ascorbyl radical and uric acid formation and inverse correlation between the ascorbyl radical formation and the hypoxanthine decomposition measured using ESR and UV spectroscopy.
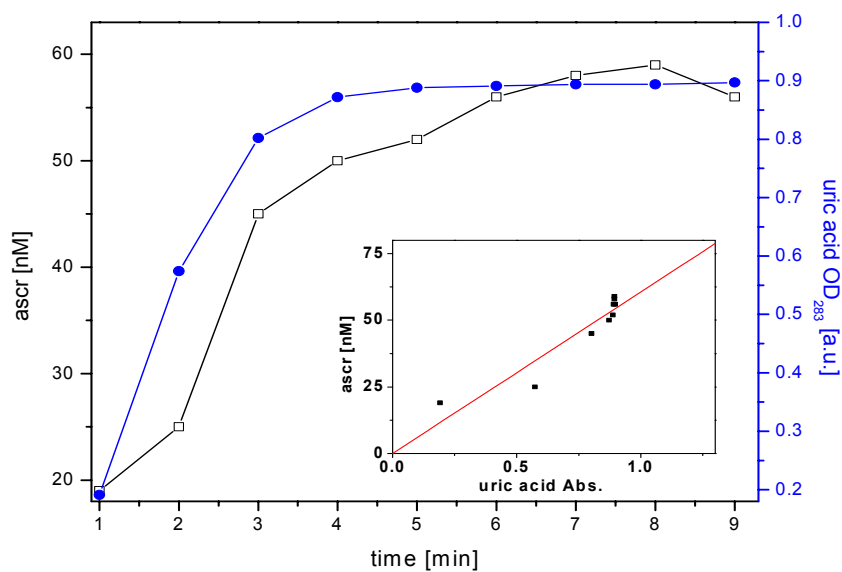
Fig. 47 *Time-dependent correlation between ascorbyl radical (–□–) and uric acid (–•–) concentrations in a hypoxantine/ xanthine oxidase system Inset: positive correlations regression between ascorbyl radical conc. and uric acid level.*
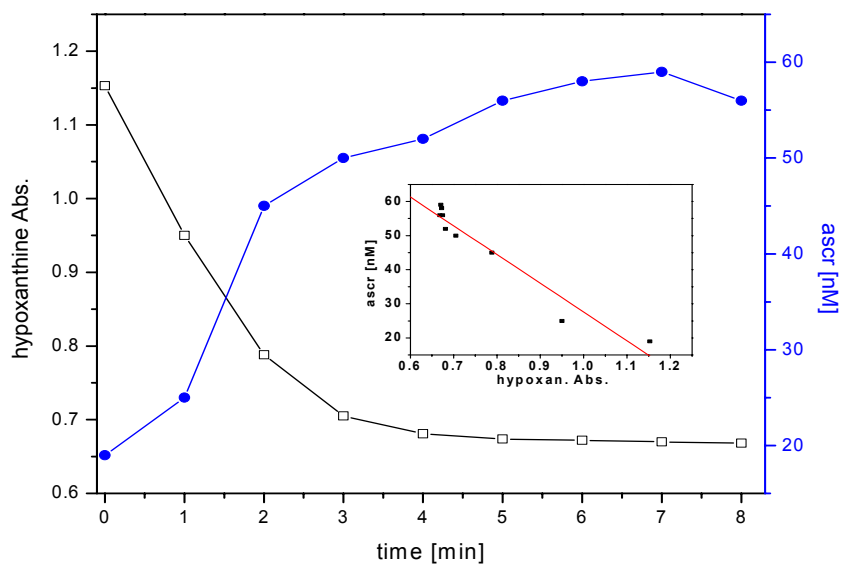
Fig. 48 *Time-dependent inverse correlation shown between an increase in ascorbyl radical concentration (–•–) and decrease in hypoxanthine concentration (–□–). Inset: negative correlation regression between ascorbyl radical conc. and hypoxanthine levels.*

Since the dependence of ascorbyl radical concentration on pH has been already established, studies on the influence of ascorbate on ascorbyl radical concentrations may help to acquire a better picture of oxidative stress. To show time-dependent free radical oxidation of ascorbate, xanthine oxidase was added to 2 levels of ascorbyl radical concentrations. Generally, ascorbate in millimolar concentrations shows background ascorbyl radical signals in untreated neutral buffers due to the presence of trace metal ions that promote ascorbate oxidation. Incubation of xanthine oxidase (XOD 1) resulted in an increase in ascorbyl radical concentrations as seen in **Figure 49**, but this reaction was relatively abrupt occurring in a time span of 2 minutes. Apparently ascorbate oxidation caused a decrease in ascorbyl radical concentrations far below its initial concentration. Further addition of xanthine oxidase (XOD 2) in this system resulted in a significant increase of the radical concentrations in relation to initial reaction condition (XOD 1). The results show that the kinetic pathway of redox reactions of ascorbate is also vastly dependent on ascorbyl radical concentrations present in the system. Therefore, experimental setups using ascorbate with low background signals tend to produce better results by studies on oxidative stress. Results show that ascorbyl radical signal intensity as a function of pH, in the presence of trace catalytic metals, oxygen levels, and ascorbate concentrations. Under these circumstances, ascorbyl radical concentrations can be determined easily when background levels are hardly visible in the ESR spectrum. This is achieved by removing traces of metal ions by passing the ascorbate medium through chelex-100 resin columns and active coal. The background ascorbyl radical signal intensities decreased significantly and baseline signal were barely visible at ascorbate concentrations below 1 mM. Therefore, by the quantitative evaluation on ascorbate oxidation *in vivo* and *in vitro* is to provide an appropriate experimental method to determine free radical concentrations.
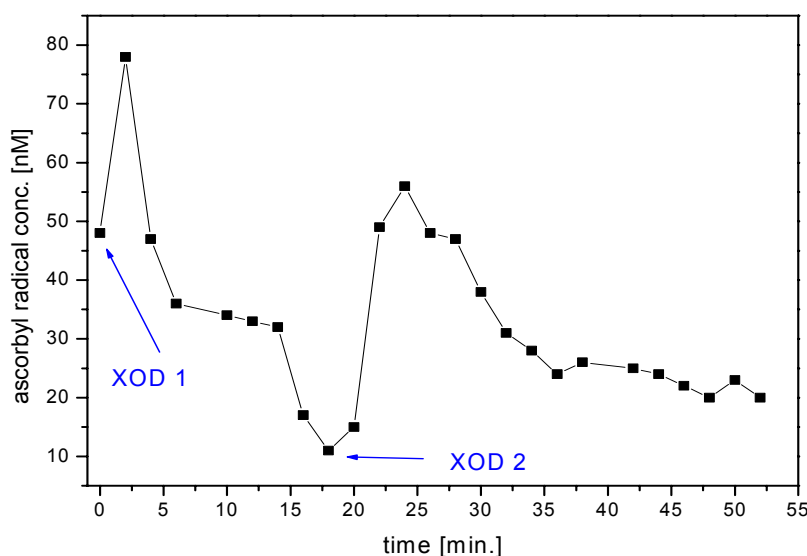
Figure 49 *Changes in ascorbyl radical concentration in xanthine PBS buffer after 2 additions of xanthine oxidase.*

3.3.3 Spin trapping methods in hypoxanthine/ xanthine-xanthine oxidase (XOD) systems

Alternative detection methods for the determination of ROS *in vivo* and *in vitro* are demonstrated using spin trapping techniques. DMPO is a widely used spin trap in which oxygen centered radicals like hydroxide and hydrogen peroxide radicals are easily trapped to form nitroxide radical adducts. These radical adducts can be detected by ESR spectroscopic methods and hyperfine splitting observed in the spectrum may give some information of the kind of radical spin adducts trapped by DMPO. However, hydrogen peroxide DMPO radical spin adducts are rather unstable and easily decomposed forming relative stable hydroxyl DMPO radical adducts. The ESR spectrum of DMPO-OH radical adducts shows a quartet with a hyperfine splitting constant of 15 G.

As in previous experiments, superoxide radical anion intermediates in xanthine/ XOD assays were determined using spin traps such as DMPO and the resulting DMPO-OOH/ DMPO-OH radical adducts formed were determined and quantified using ESR spectroscopy (**Figure 49)**
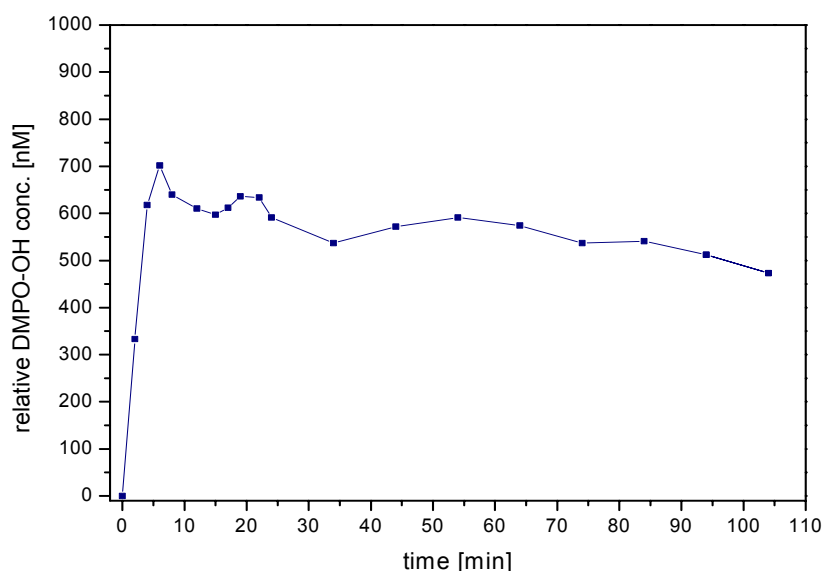
Figure 50 *Increase in nitroxide radical concentration of DMPO-OH adduct in the xanthine, xanthine oxidase system in PBS during a time period of 105 minutes.*

Incubation of DMPO in xanthine / xanthine oxidase in PBS buffer caused an increase in DMPO-OH spin adduct concentration. Time-dependent studies show the involvement of oxygen free radical intermediates. Data in **Figure 50** show an increase in nitroxide concentrations after 6 minutes that remained relatively stable even after 100 minutes. **Figure 51** show non-linear kinetics with cross-correlation between xanthine oxidation and DMPO-OH formation.
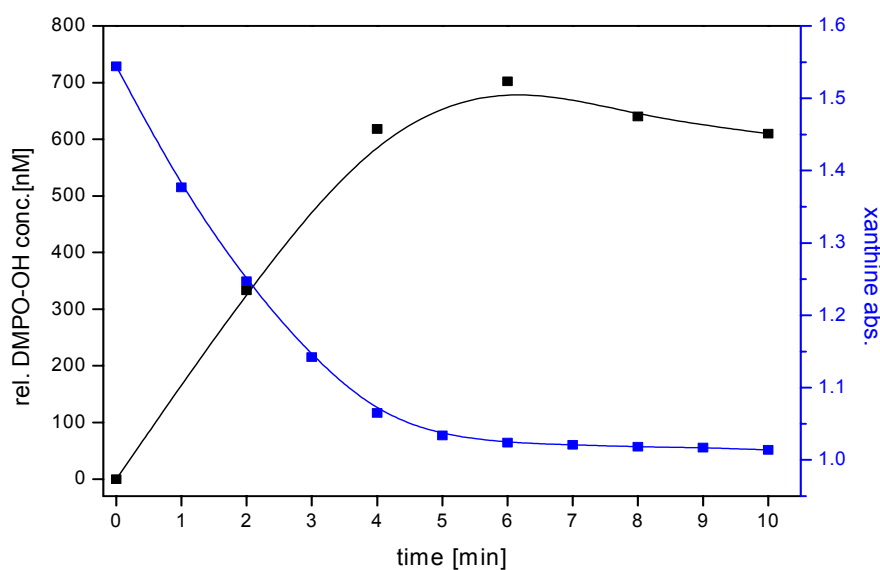
Figure 51 *Cross-correlation between spin trap nitroxide radical concentrations (DMPO-OH) (–■–) and xanthine levels (–●–) against time after the introduction of xanthine oxidase in PBS at pH 7.4.*

In summary, superoxide radical anions which were efficiently generated from xanthine/xanthine oxidase complexes were detected using ascorbate and DMPO spin traps. From free radical oxidation patterns under similar conditions, xanthine or hypoxanthine breakdown and free radical generation could be established using UV and ESR spectroscopy with fairly good correlation. It can be concluded that further studies on ascorbate oxidation and spin trapping in the presence of free radicals could be used to determine oxidative stress *in vitro*.

3.3.4 Vitamin C and vitamin E interactions in biphasic systems

Vitamin C and vitamin E (α-tocopherol) are naturally occurring antioxidants in biological systems. Vitamin E plays a vital role in the prevention of free radical-mediated oxidation of low-density lipoproteins believed to be major factor in the development of atherosclerosis. Vitamin E, a lipophilic antioxidant is mostly located on lipid membrane surfaces and in lipoproteins. Studies on the interaction of vitamin C, a water-soluble antioxidant, and vitamin E in biological systems may show some interesting redox properties of these antioxidants

during periods of oxidative stress[schnieder et al 2003]. The cooperation between ascorbate and α-tocopherol, allow the oxidized α-tocopherol radical in lipid phase is to be reduced back by ascorbate to α-tocopherol, in the cytoplasm. The ascorbyl radical can spontaneously disproportionate, or be reduced by cellular enzymes. In summary, free radicals originating from the lipid membrane can be removed from the system using two different anti-oxidants.

To asses the possible reactions of α-tocopherol and ascorbate interaction in biphasic systems, experiments were conducted in aqueous/ ethyl acetate medium. Since the oxidation of the lipophilic α-tocopherol is not favourable in aqueous medium, 10 % Triton-X 100 detergent was added forming an α-tocopherol suspension. For the generation of α-tocopherol radical, Fenton's reagent was added as source of oxygen radicals. The oxidized α-tocopherol radical was extracted with ethyl acetate and shaken vigorously with 10 mM ascorbate in aqueous solution for some time. Kinetic correlation measurements were conducted using ESR spectroscopy to study redox reactions of α-tocopherol radical and ascorbyl radicals in biphasic systems.
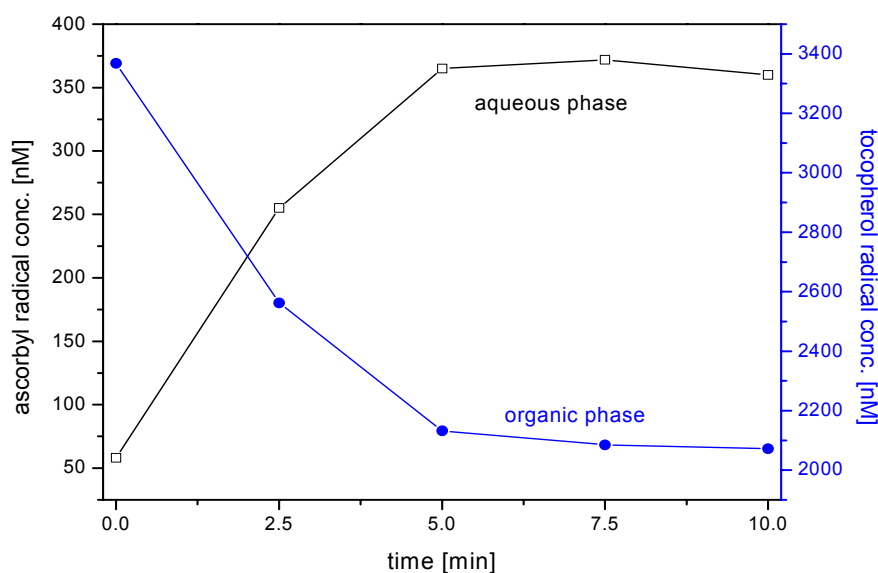
Figure 52 *The decrease of tocopherol radical concentration in organic medium (−●−) and the increase in ascorbyl radical concentrations (−□−) in aqueous medium after vigorous shaking of both immiscible mixtures.*

The results in **Figure 52** show cross-correlations with a 10-fold depletion of α-tocopherol radical in the organic phase and a 3-fold increase in ascorbyl radical concentrations in aqueous solution over a period of 10 minutes. This interesting phenomenon of one-electron redox transfer from α-tocopherol to ascorbate might be used as model systems to show similar cascades of recycling processes taking place to eliminate radicals in biological systems.

3.3.5 Ascorbate and spin trapping studies on oxidative stress observed under ischemia/anoxia and reoxygenation conditions.

Over the past decade direct observations on the production of ROS produced during ischemia and reperfusion has been determined using ESR spectroscopy with spin trapping techniques. These methods showed an increase in ROS production during periods of ischemia, but even more during periods of reoxygenation accompanied by a sudden burst of free radical species. Identifying the types of free radicals was accomplished by determining the hyperfine splitting constants of spin trapped radical adducts. An additional method for the identification and quantification of free radicals was carried out using ascorbate for studying oxidative stress. Moreover, ascorbate as a non-toxic antioxidant can be easily monitored over a broad range of concentrations without showing any toxic effects in cells.

In order to study oxidative stress under normoxia, hypoxia and reoxygenation *in vitro*, ascorbate oxidation was monitored with the help of ESR spectroscopy. Human colon carcinoma cell (RKO) cultures were incubated in DMEM serum medium at 37°C. Prior to the experiment, cells were treated with 1 mM ascorbate and incubated under normoxia, hypoxia, anoxia and reoxygenation. Subsequently, under these experimental conditions ascorbyl radical concentrations were determined after 30, 60, 90 and 120 minutes (**Figures 53** to **56**). During 60 minutes ischemia and hypoxia a marginal increase in ascorbyl radical concentrations was observed that increased significantly after 30 minutes reoxygenation. However, under normoxia an increase in ascorbyl radicals were seen after 60 minutes. Therefore, the overall trend was a marginal increase in ascorbyl radical concentrations during ischemic and normoxic conditions and a significant increase after reoxygenation.
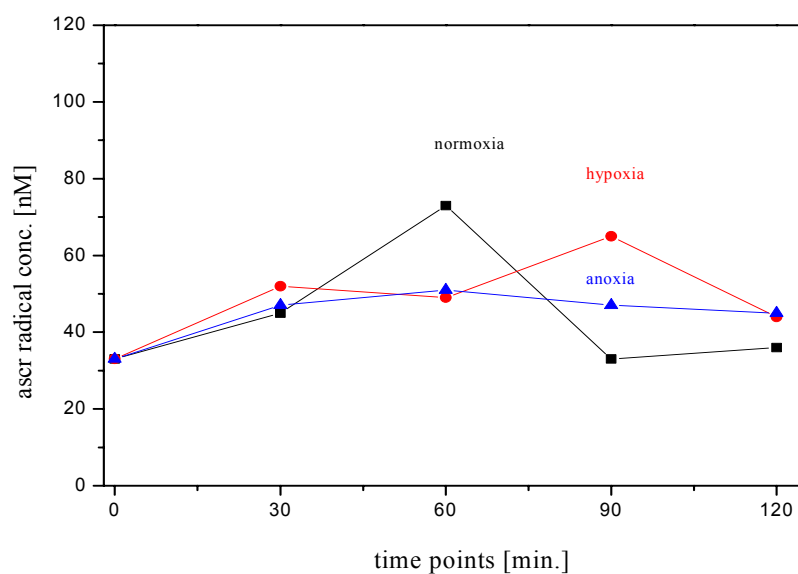
Figure 53 *Changes in ascorbyl radical concentrations under normal (–■–), anoxia (–▲–) and ischemia (hypoxia) (–●–) conditions treated with 1mM ascorbate in RKO cell lines during a period of 120 min.*
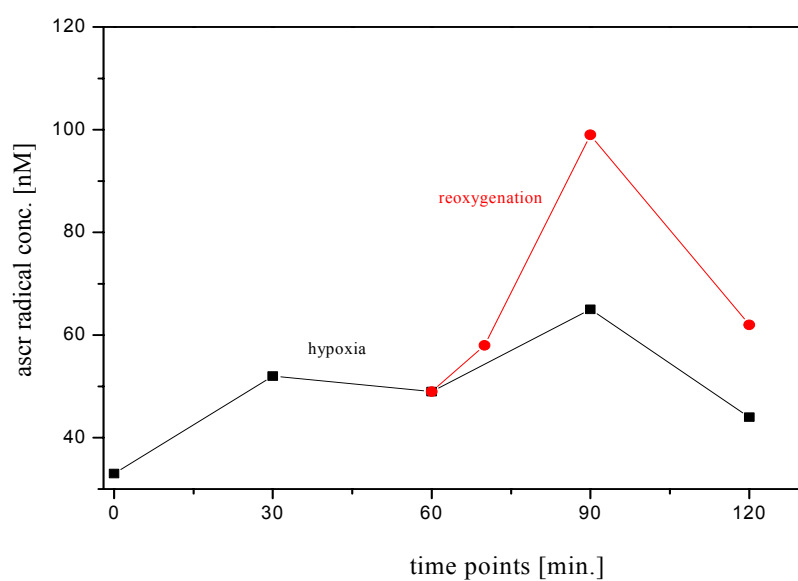
Figure 54 *Ascorbyl radical concentrations in RKO cells treated with 1 mM ascorbate under ischemia (–■–) and reoxygenation (–●–).*
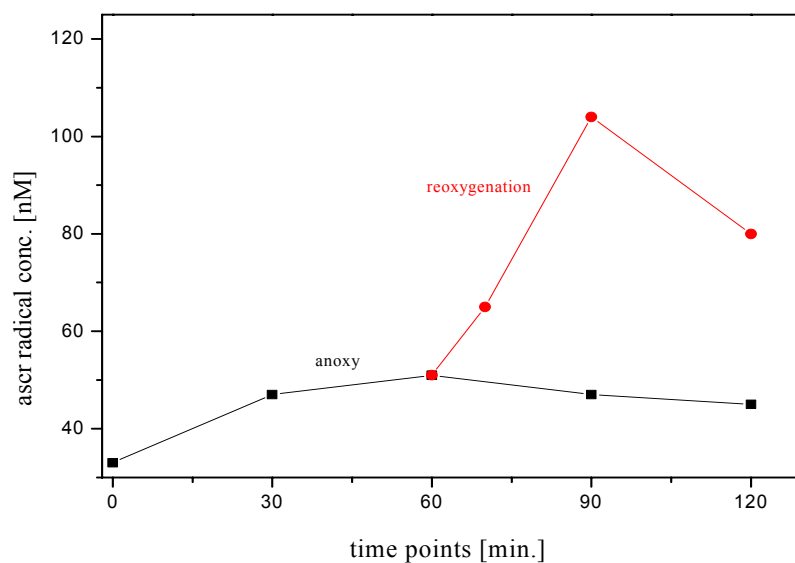
Figure 55 *Ascorbyl radical concentrations in RKO cells treated with 1 mM ascorbate under anoxia (─■─) and reoxygenation (─●─).*
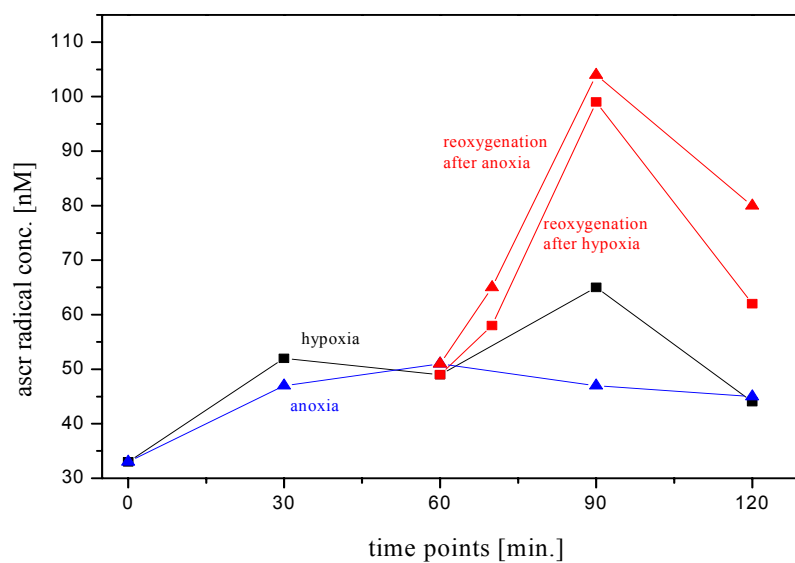
Figure 56 *Comparing figures 41 and 42 in studying non-linear changes of ascorbyl radical concentrations in RKO cell under anoxia (─▲─), ischemia (─■─) and reoxygenation (─■─ , ─▲─).*

Since ascorbate is a major water-soluble antioxidant showing little or no toxic effects at high concentrations, it was an ideal choice as a natural non-invasive marker of free radical oxidations taking place in biological systems. *In vitro* studies showed time-dependent ascorbate oxidation in RKO cell lines during hypoxia, anoxia and reoxygenation.

Interestingly, ascorbyl radical reactions during periods of oxidative stress revealed similar kinetic patterns in healthy subjects and for patients after reperfusion and surgery as described in the former chapters. Generally, an increase in ascorbyl radical concentrations was observed after a 30-minute period of ischemia and reoxygenation. Therefore, observations of ascorbyl radical reactions *in vivo* and *in vitro* suggest the efficacy of ascorbate for the determination of oxidative stress.

An alternative strategy for determination of ROS *in vitro* was the application of spin trapping methods with the help of ESR spectroscopy. This indeed is considered by some authors to be superior to ascorbate, because, analytical and quantitative results can be achieved with experiments on spin traps. In the following experiments, the involvement of spin traps to determine radical species during anoxia, ischemia and reoxygenation is considered and compared with ascorbyl radical reactions. The important criterion for the selection of a good nitrone spin trap in biological investigations is to know about its cellular penetration and resistance to metabolism, specificity towards the free radical of interest, and slow decay rate of the generated nitroxide. To evaluate free radical species during ischemia and reoxygenation, DMPO was considered a spin trap of choice. DMPO was incubated in RKO cell medium following the same experimental procedure as with ascorbate. Paradoxically, all the collected spin trapped probes were ESR silent showing no nitroxide signals even after increasing its concentrations in the cell medium. To assess for loss of ESR signal intensity, kinetic studies were conducted with DMPO in serum free and serum DMEM medium.

DMPO-OH spin adducts were obtained by reacting hydrogen peroxide and ferrous ion in serum free DMEM medium. As shown in **Figure 57,** kinetics of DMPO-OH radical adducts was determined by ESR spectroscopy. Based on kinetic observations of DMPO-OH decomposition, depletion of nitroxide concentrations followed first-order exponential decay with a half-life period of 18 minutes. However, as the above experiments were repeated in serum DMEM medium, nitroxide signal intensity of DMPO-OH disappeared rapidly with half-life of 3 minutes. From these kinetic observations, it can be assessed that FBS serum in DMEM medium enhanced paramagnetic nitroxide decomposition significantly (**Figure 58**).

Therefore, spin trapping experiments with DMPO carried out in FBS DMEM medium were inadequate for studies on ischemic and reoxygenation using RKO cells.

Since experiments carried out in serum-containing medium were unsuccessful, further investigations were carried out using serum-free Quantum 263 medium under similar experimental conditions. On one hand kinetic curves showed that nitroxide radicals were relatively stable, but on the other hand RKO cells showed retarded growth and apoptosis. Therefore, spin trapping of ROS in biological systems carried out in serum free Quantum 263 medium was also inadequate to estimate oxidative stress during ischemia and reoxygenation (**Figure 59**).
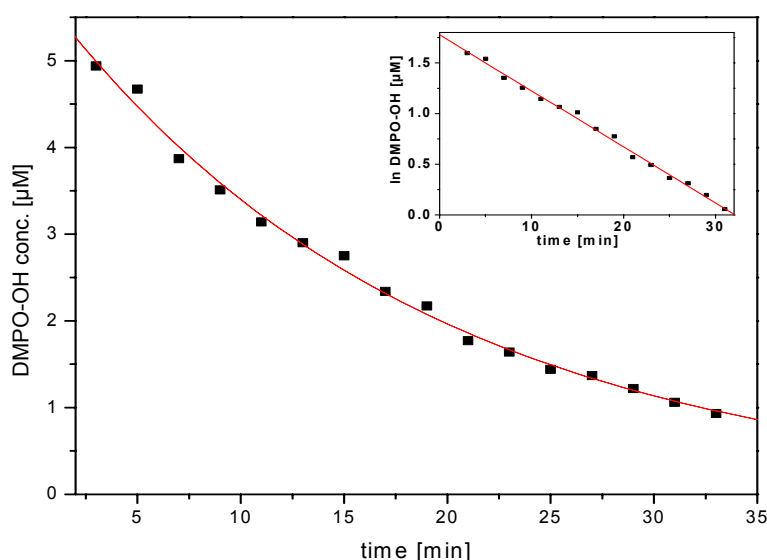
Figure 57 *1st -Order decrease of DMPO-OH signal intensity in DMEM medium at pH 7.8 with a half life time of 18.3 minutes. Inset: Verification of first-order equation by plotting ln DMPO-OH conc. versus time.*

In summary, ascorbic acid was seen to be an excellent scavenger of free radical oxygen species during ischemia and reoxygenation. An increase in ascorbyl radical concentration was seen during reoxygenation after anoxia and hypoxia. However similar experiments determined with the help of spin traps were not successful.
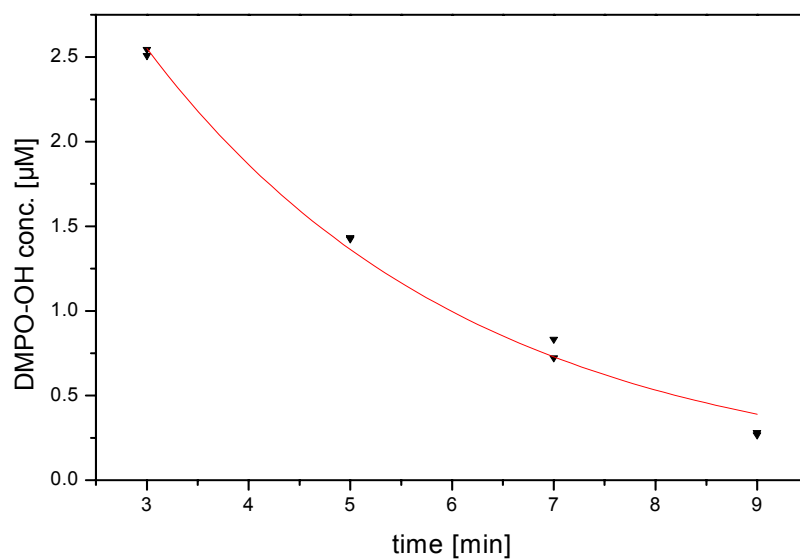
Figure 58 *The decrease in 100 mM DMPO radical adduct of the 1ˢᵗ-Order in DMEM serum medium at pH 8.3, with a half life of 3.2 minutes.*
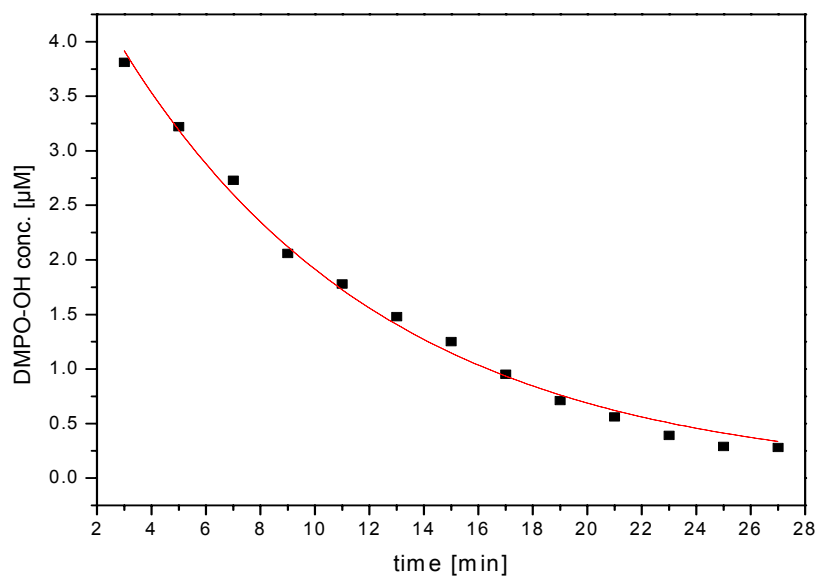
Figure 59 *The decrease in 100 mM DMPO radical adduct in serum-free Quantum 263 medium at pH 7.8 with a half life of 9.77 minutes.*

3.3.6 Nitroxide activity of fluorescent-labelled spin probes in the presence of ROS

New double sensors involving fluorescent dansyl derivatives and paramagnetic pyrrole-nitroxide derivatives were developed in the past and applied in biological systems as indicators of oxidative stress [kalai, Hideg et al 2002]. It has been suggested that the paramagnetic nitroxide radical fluorophore, is reduced to hydroxyl amine derivatives with Fe^{2+} as catalyst. This reaction deserves special attention, since time-dependent patterns are observed with ESR as well as fluorescence spectroscopy. Paramagnetic nitroxide groups attached to fluorophores are shown to exhibit characteristics of fluorescence quenching. Therefore, reactions of fluorescent labelled spin probes in the presence of reactive oxygen species can be investigated using ESR as well as fluorescence spectroscopy [Hideg et al]. Correlations between fluorescence quenching and nitroxide reduction may reveal some information about the involvement of ROS *in vivo* during periods of oxidative stress.
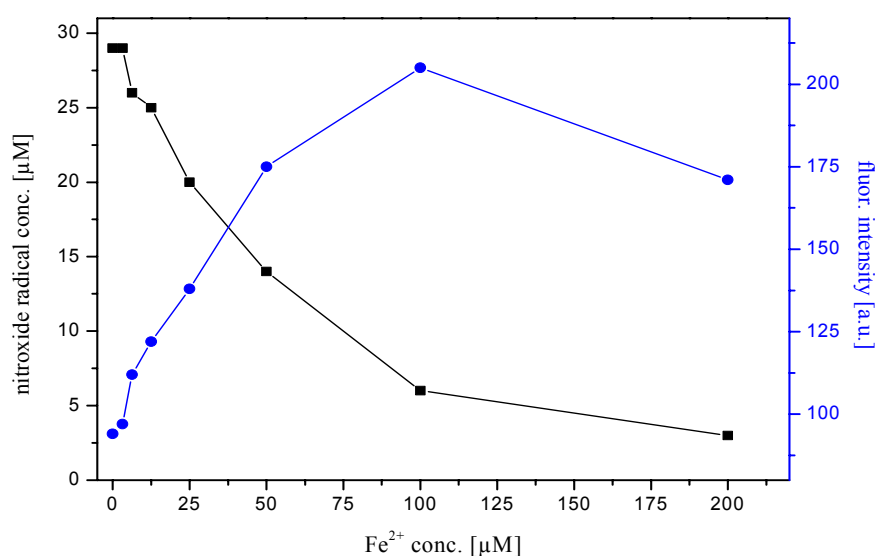
Figure 60 *Cross correlation between nitroxide reduction (–■–) and fluorescence intensity (–●–) from a spin label-dansyl derivate with a wide range of Fe^{2+} concentrations.*

To study the effects of nitroxide reduction and fluorescence quenching, 50 µM dansylamide spin label was added to various ferrous II ammonium sulphate concentrations in PBS medium. Results in **Figure 60** show the influence of increasing Fe^{2+} concentrations on nitroxide levels and fluorescence intensity. The ferrous-ion-dependent nitroxide reduction and fluorescence intensity showed inversely correlated nonlinear curves. Interestingly, at Fe^{2+}

concentrations higher than 100 μM, a decrease in fluorescence intensity was observed. It can be assumed that oxidation reactions at the aromatic ring results in the breakdown of conjugated systems. Apparently, addition of Fe^{2+} concentrations higher than 100 μM to spin labelled fluorophores may lead to artefacts. Therefore, studies on fluorescence quenching deserve special attention at metal-ion concentrations far exceeding physiological values.

The two-faced character of fluorescent labelled nitroxide determined by ESR and fluorescence spectroscopy can be considered for *in vivo* investigations in the near future. Moreover, their involvement in life-time measurements using fluorescence confocal laser microscopy to determine site related oxidative stress in living organisms may be of some significance. Therefore, methods are to be proposed for the synthesis of a new fluorescent-labelled ascorbic acid, which can turn out to be an efficient indicator for free radicals in biological systems.

3.4 Fluorescent-labelling of ascorbic acid with N-methylisatoic anhydride

In the last decade, extensive studies on oxidative stress using ESR spectroscopy were achieved with the help of spin trapping techniques to detect and identify free radicals in biological systems. However, numerous studies on spin trapping previously and in this work showed its limitations in the determination of free radicals. Spin trapped radical adducts decomposed rapidly during *in vivo* studies and artefacts observed may presumably complicate the assessment of free radicals.

An alternative method for the determination of radical intermediates is to develop methods for the synthesis of new fluorescence labelled ascorbic acid derivates. These antioxidant fluorescent labels may open new pathways in determining free radical species using ESR as well as fluorescence spectroscopy, in which the latter may overcome the detection limits of ESR spectroscopy. Moreover, due to reduced (almost no toxic capacity) toxicity of ascorbic acid, live-time measurements in the detection, interaction and co-localization of ROS using fluorescence confocal laser microscope can be achieved in the near future.

3.4.1 Acylation of hydroxyl groups of ascorbic acid with DMAP as catalyst in organic solvents

N-methylisatoic anhydride (IA) (**1** in **Figure 61**) was considered an ideal choice as a fluorescent label for the synthesis of ascorbic acid ester. The N-methylanthranilate group is one of the smallest known fluorophores that emit relatively intense fluorescence upon UV excitation.

Moreover it reacts with nucleophiles like alcohols at its oxazine ring with the influence of base catalyst forming anthranilate ester derivatives at ease. For the synthesis of N-methylanthranilate group analogs, 4-dimethylaminopyridine (DMAP) (**3**) was chosen as a hyper-nucleophilic acylation catalyst on less reactive alcohols under basic conditions. DMAP has two distinct advantages as a catalyst; it enhances the yield and rate of reactions and it allows sensitive reactions to be carried out under milder conditions thereby reducing unwanted side effects. Searching for a method to label ascorbic acid that would not hinder its antioxidant functional capacity is to acylate the less reactive 6-OH (**6d**) position. This may prove to be relatively persistent, since the ene-diol at positions 2-OH (**6a**) and 3-OH (**6b**) on the lactone ring of ascorbic acid is more accessible to nucleophilic attack. The catalyst DMAP

may make this process easier by forming acyl pyridinium ion intermediates (**3**) at position 5-OH (**6c**) or 6-OH (**6d**) and therefore easily accessible to acylation.

Based on the collected information, a novel approach for the synthesis of fluorescent-labelled ascorbic acid esters was achieved by stirring equimolar ascorbic acid (**2**) and N-methylisatoic anhydride (**1**) with DMAP (**3**) in DMF. Triethylamine, an organic base was added to remove the intermediate acid formed during the reaction and the mixture was allowed to be heated up to 65 °C for 3 h until carbon dioxide evolution ceased. The solvent was evaporated and the raw product dissolved in a basic medium containing sodium carbonate solution. The aqueous mixture was allowed to stand for 10 minutes and was subsequently re-acidified with diluted phosphoric acid. With this method, acyl groups tend to migrate in the direction away from the neighbouring carbonyl group and interact with hydroxyl groups positioned within the lactone side chain [Nomura & Sugimoto 1965, Reese & Trentham 1966]. The ascorbate ester at neutral pH was re-acidified to form ascorbic acid ester, which was eventually extracted with an organic solvent and evaporated under vacuum.

The possible mechanism for the acylation of alcohols is the formation of an intermediate acyl pyridinium ion (**4**) by the reaction of DMAP (**3**) with N-methylisatoic anhydride (**1**) (**Figure 61**). This step is more rapid than for pyridine because DMAP is a stronger base due to the electron donating effect of the p-dialkylamino group. With alcohols as nucleophiles and the organic base triethylamine, DMAP is released from its salts leading to nucleophilic attack of alcohols (**2a-d**) to form anthranilate esters (**6**).
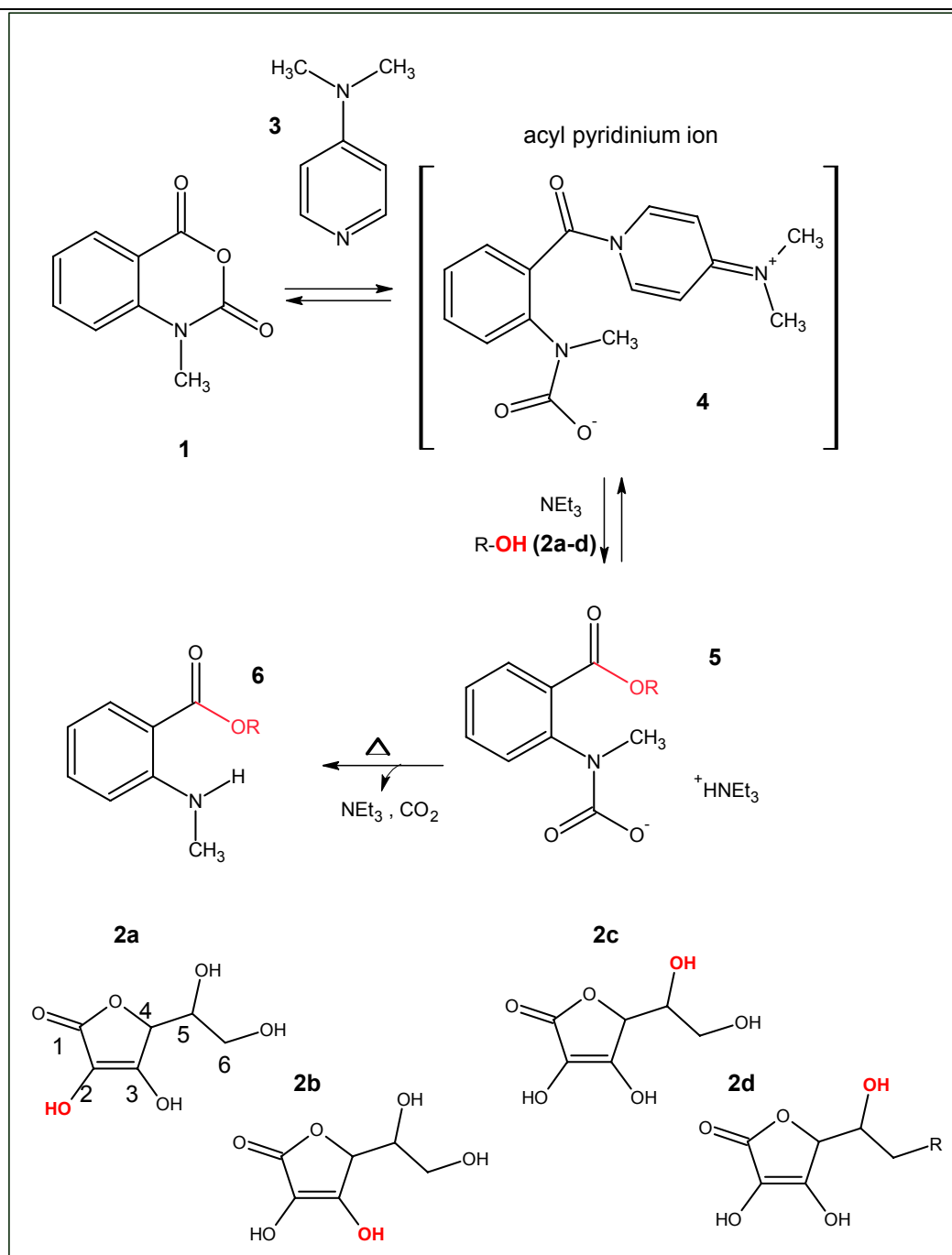
Figure 61 *The possible mechanism for the acylation of alcohols (**2a-d**) is the formation of a relative stable intermediate acyl pyridinium ion (**4**) by the reaction of 4-dimethylaminopyridine (DMAP) (**3**) with N-methylisatoic anhydride (**1**). The second step is promoted by the presence of the strong organic base triethylamine that is known to release DMAP from its salts.*

3.4.2 Thin layer chromatography (TLC), HPLC and MPLC

As a result of competing reactions of the fluorescent label at positions 2-OH (**2a**) and 3-OH (**2b**) on the lactone en-diol ring with 5-OH (**2c**) and 6-OH (**2d**) on the lactone side chain of

ascorbic acid to from esters, fluorescent label ascorbic acid ester derivatives were determined by the application of thin layer chromatography. The ester derivatives were eventually separated by HPLC for qualitative analysis and MPLC for quantitative separations.

Reversed phase thin layer chromatographic (TLC) plates were applied for pre-analytic separations of various fractions. The raw product extracted with ethyl acetate was dissolved in acetonitrile and two TLC tests were conducted before and after extraction with ethylacetate with acetonitrile water mixture (30:70) as moving phase (**Figure 62**). The separated fractions from the raw product extract were observed under a UV-lamp with excitation wavelength of 254 nm and 366 nm respectively (**Figure 63**).
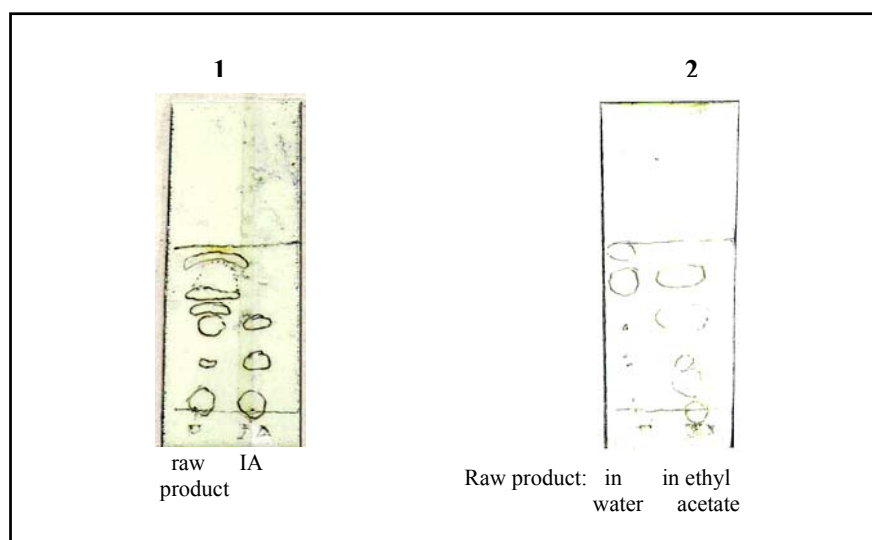
Figure 62 *TLC-RP 18, raw product and N-metyl isatoic anhydride (IA) (**1**) with a moving phase of 30: 70 % acetonitrile/ water. After the extraction of the raw product with ethyl acetate (2nd lane) from water (1st lane) (**2**).*

Fractions of the raw product on the TLC plate showed a wide range of R_f values in relation to the educts (**Figure 63**). Raw fractions produced larger R_f values (0.93 and 0.67) than the less polar N-methylisatoic anhydride (R_f 0.53).
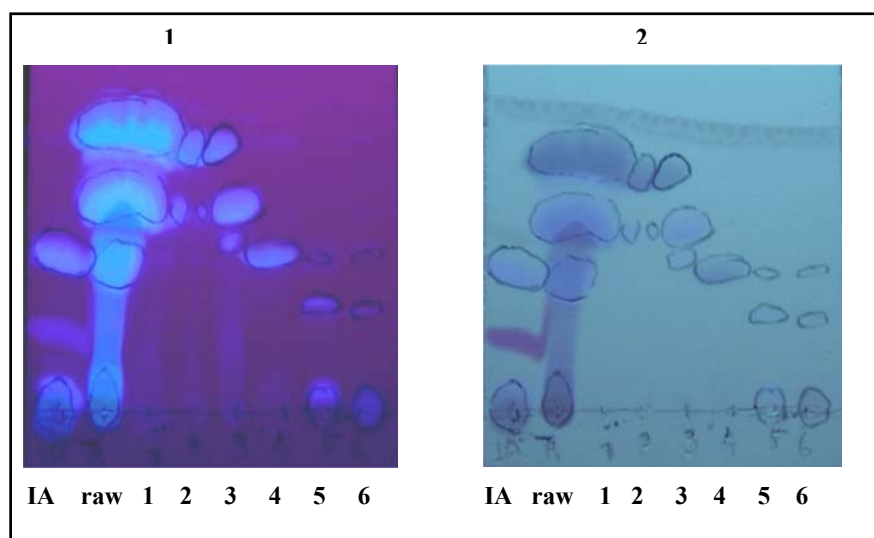
Figure 63 *TLC-RP 18 of raw product* ***(raw)****, N-methyl isatoic anhydride* ***(IA)*** *and fractions* ***1-6*** *with a moving phase of 30:70 % acetonitrile and water. The TLC-plates were observed under UV at 333 nm (**1**) and 254 nm (**2**).*

Since TLC separation methods using reversed-phase were relatively successful, the high-performance liquid chromatographic (HPLC) method was chosen as a subsequent strategy to separate the fractions of the raw product. A reversed-phase column was chosen for separation using acetonitrile and water as moving phase. Therefore, chromatographic separation of
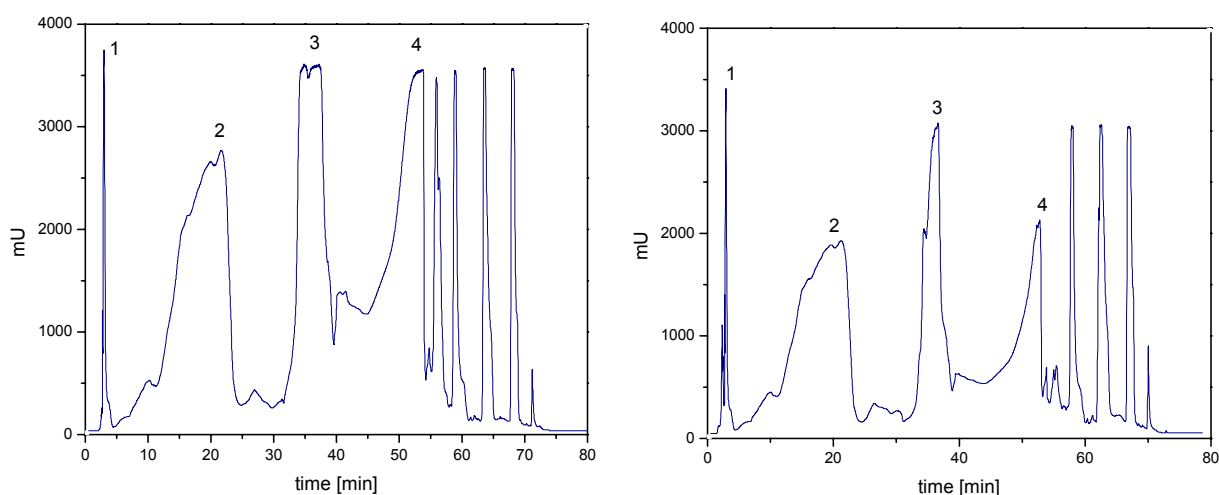
Figure 64 *Reverse-phase HPLC with a UV detector set at 357 nm* **(left)** *and at 277 nm* **(right)** *showing the fractions after elution using water and acetonitrile as moving phase.*

the raw product was achieved by passing it through a reversed- phase 250 mm HPLC column. The fractions were separated using water/acetonitrile as eluent with a flow rate of 1ml/min and monitored by a UV-detector set at 277 and 357 nm (**Figures 64**).
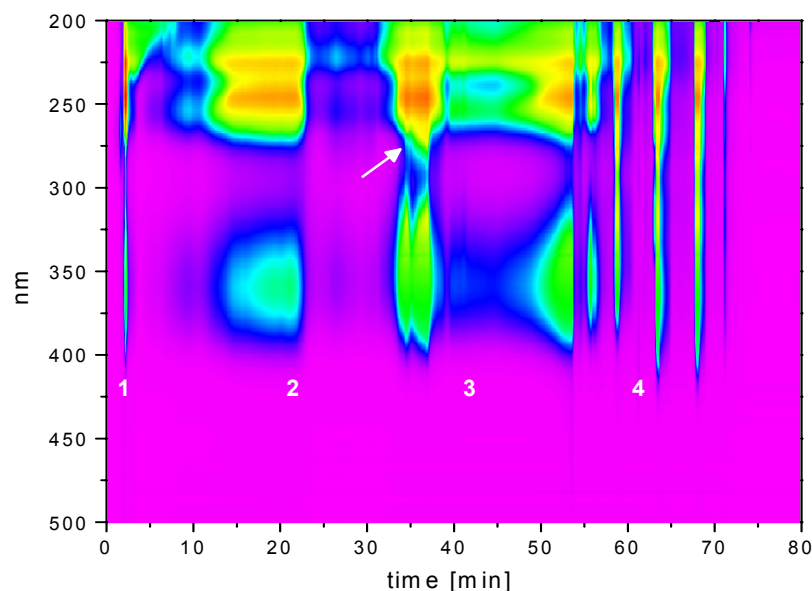
Figure 65 *A 2D spectrum of fractions 1 to 4 separated from a reverse-phase HPLC column. Protruding absorptions band (arrow) from fraction 3 of ascorbic acid derivative detected at 265 nm.*
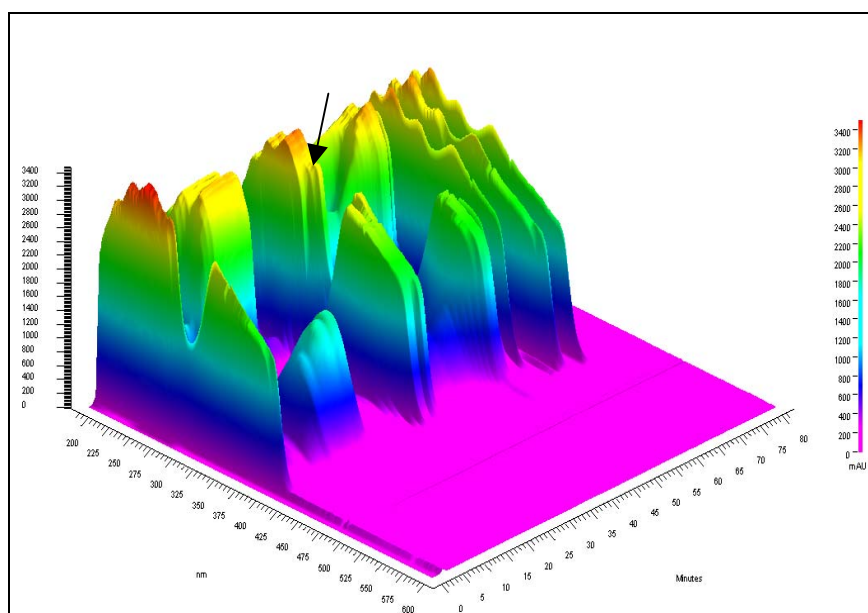
Figure 66 *The same HPLC elution's spectrum as above with a 3D view. A more detailed view of ascorbic acid derivative (arrow), showing a shoulder of the absorptions band.*

Also, a 2 D and a 3 D spectrum is presented in **Figures 65** and **66** to highlight the absorption band of ascorbic acid at 265 nm protruding out in fraction 3. Furthermore, 2D and 3D spectra

showed absorption bands of ascorbate and N-methylisatoic anhydride, so that additional information could be gathered from these fractions. As for the purification of fractions after HPLC separation of the raw product, they were collected and lyophilized overnight. The fractions 2 and 3 gave yields of 32 wt. % and 21 wt. % respectively.

However, it is hardly possible to quantitatively determine the fractions through HPLC methods due to insufficient yield. Therefore, further quantitative separations were achieved through MPLC techniques. Here, a larger column of middle pressure liquid chromatographic column (MPLC) was exchanged for an HPLC. The UV-detector was set at 277 nm with a flow rate of 2 ml/min and showed separation peaks similar to those of HPLC. Dried fractions were characterized analytically by ^{1}H-NMR, Mass, UV, ESR and fluorescence spectroscopy.

3.4.3   1H-NMR spectroscopy of the fractions 2 and 3

For the structural determination of fractions 2 and 3 after chromatographic separations, ^{1}H-NMR spectra were taken of the fractions dissolved in D_6-DMSO and $CDCl_3$. The data obtained from the ^{1}H-NMR spectra were studied and compared with the educts.

Subsequently for the educt N-methylisatoic anhydride **(Figure 67)**, the chemical shifts in $CDCl_3$ of aromatic protons appeared at low field between 8.18 − 7.20 ppm and the high field proton signal at 3.61 ppm could be identified as N-methyl group. By the characterization of ascorbic acid in D_6-DMSO (**Figure 68**), the protons at the ene-diol lactone ring showed characteristic resonance signals at low fields of 11.01 and 8.30 ppm respectively. The resonance signal of the proton at the lactone ring at position C4 appeared at 4.72 ppm. The chemical shifts of the two hydroxyl groups on the lactone side chain at positions C5 and C6 overlapped each other resulting in a broad signal at 4.87 ppm and the 3 protons attached to the carbon side chain at positions C5 and C6 showed signals at 3.73 and 3.44, ppm respectively. The NMR spectrum of ascorbic acid was identical to literature values obtained from Integrated Spectral Data Base System (SDBS) for Organic Compounds by the National Institute of Advanced Industrial Science and Technology, Japan.
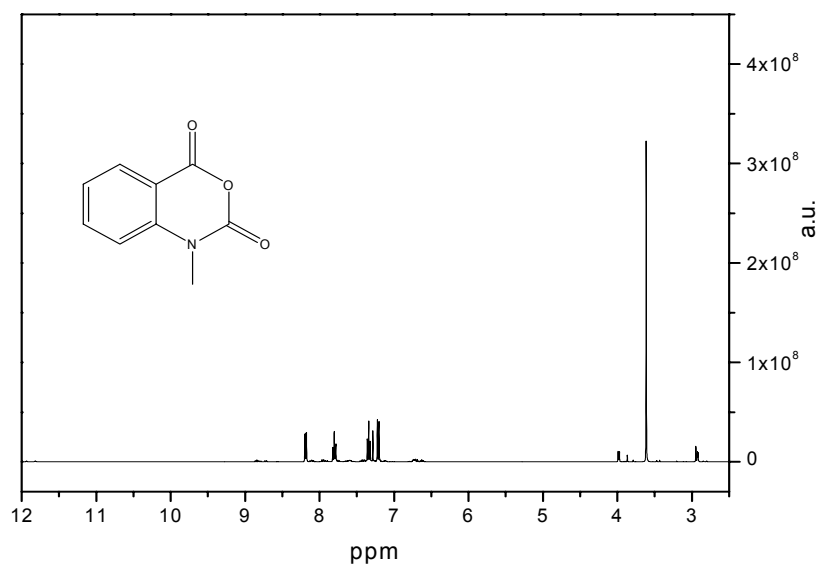
Figure 67 *^{1}H-NMR 400 MHz, N-methylisatoic anhydride in CDCl₃, 4*CH-aromatic ring (m) 8.18-7.20 ppm, N- CH₃ (s) 3.61 ppm.*
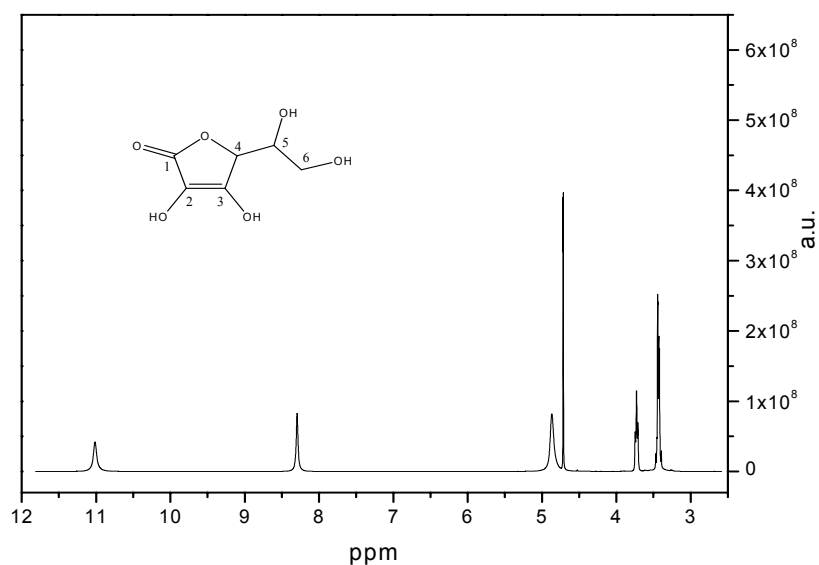
Figure 68 *^{1}H-NMR 400 MHz, Ascorbic acid DMSO-D₆, 2-OH (s) 11.01 ppm,3-OH (s) 8.30 ppm, 6-OH (s) 4.87ppm, 4-CH (d) 4.72 ppm, 5-CH (t) 3.73 ppm, 6-CH₂ (m) 3.44 ppm.*

For the characterization of the fractions with ^{1}H-NMR spectroscopy, fractions 2 (**Figure 69**) and 3 (**Figures 70 and 71**) were investigated in D₆-DMSO and CDCl₃ solvents respectively.
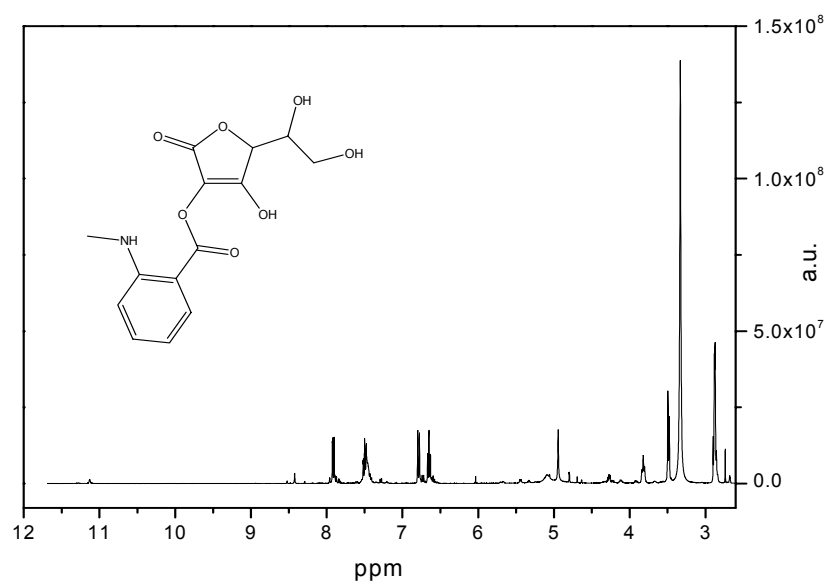
Figure 69 *^{1}H-NMR 400 MHz, Fluorescent- labelled ascorbic acid of fraction 2 in DMSO-D$_6$, 4*CH-aromatic ring (m) 7.92-6.65,2* OH (s) 5.08 ppm, CH (s) 4.94 ppm, CH (t) 3.82 ppm, CH$_2$ (d) 3.48 ppm, N-CH$_3$ (q) 2.87ppm.*

The ascorbic acid derivate of fraction 3 (**Figure 69**) in D$_6$-DMSO showed characteristic en-diol resonance signals at low fields of 11.13 and 8.43 ppm respectively. However, ^{1}H-NMR resonance peaks of the hydroxyl groups of ene-diol were not seen in CDCl$_3$ (**Figure 71**) which may suggest proton exchange with the deuterated solvent. Chemical shifts between 8.0 and 6.5 ppm identified, as aromatic proton peaks were present in both the deuterated solvents. The resonance signal of the 4-CH atom on the lactone ring and the 5-CH, 6-CH$_2$ (**Figures 70 and 71**) atoms on the side chain were shifted towards lower field with an increasing X-CH$_n$ (X = 4, 5, and 6) numerical order (**Table 24**) as compared to CH proton signals of ascorbic acid (**Figure 68**). Presumably, this is a consequence of deshielding, due to the proximity of the ester group, which largely influences the 6-CH$_2$ and 5 CH-OH protons resulting in chemical shift to lower field. Chemical shifts of the N-methyl amino group on the aromatic ring were identified at high field at 2.9 ppm present in both deuterated solvents.
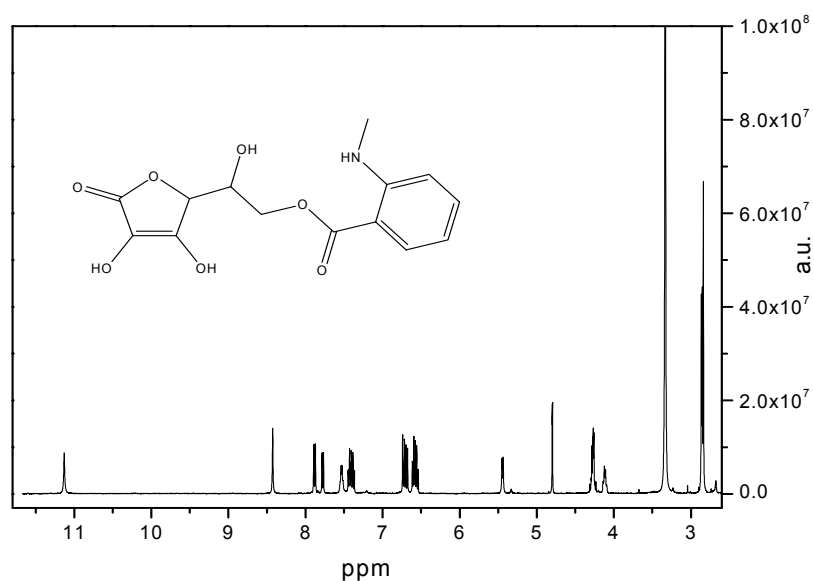
Figure 70 *^{1}H-NMR 400 MHz, Fluorescence labelled ascorbic acid product of fraction 3 in DMSO-D$_6$, OH (s) 11.13 ppm, OH (s) 8.43 ppm, CH-aromatic ring (m) 8.43-6.56 ppm, OH (d) 5.45ppm, CH (s) 4.8 ppm, CH$_2$ (m) 4.27 ppm, CH (q) 4.14 ppm, N-CH$_3$ (d) 2.85 ppm.*
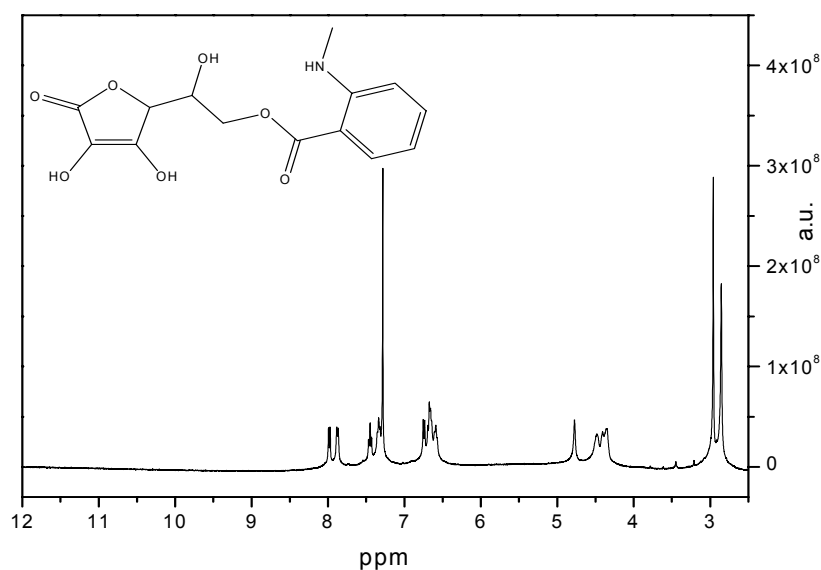
Figure 71 *^{1}H-NMR 400 MHz, Fluorescence labelled ascorbic acid product of fraction 3 in CDCl$_3$, CH-aromatic ring (m) 7.9-6.67, -OH (s) 4.78, CH (s) 4.49, CH$_2$ (d) 4.3, N-Me (d) 2.89.*

However, in the characterization of fraction 2 (**Figure 69**), the absence of ene-diol proton peaks at low field in relation to fraction 3 and ascorbic acid were taken into consideration.

This indicates the acylation at 2 C-OH or 3 C-OH of the ene-diol group at the lactone ring of ascorbic acid. Moreover, proton resonance peaks at position C4, C5 and C6 showed no evidence of deshielding which may have been influenced by acylation at these positions if they had occurred.

In a short summary, special attention was given to ene-diol and side chain protons of the fluorescent-labelled ascorbic acid for the determination and characterization of fractions 2 and 3 using ^{1}H-NMR spectroscopy (**Figures 69** to **71**).

Absence of chemical shifts of fraction 2 at low fields revealed acylation at the ene-diol group as seen in **Figure 69**. Moreover, protons on the side chain in fraction 2 were hardly influenced by electronegativity, signifying the absence of a carbonyl group in its vicinity. Therefore, this may rule out acylation at positions 5 CH-OH or 6 CH$_2$-OH on the side chain of the lactone ring. Whereas for fraction 3 (**Figure**s **70** and **71**), chemical shifts of the ene-diol group were seen at low field similar to ascorbic acid resonance signals. Apparently the protons on the short carbon side chain of ascorbic acid derivate were significantly affected by de-shielding, resulting in low field chemical shifts predominantly observed at protons positioned on the 6th carbon atom (see **Table 24**).

These results suggest acylation reactions were successfully achieved in the synthesis of fluorescent-labelled ascorbic acid at positions C-2, C3, C5 or C6 (**Figure 61**). Acylation at position C2 or C3 are not relevant for this study, since ester formation at these positions blocks the antioxidant capacity of ascorbic acid. Conversely, acylation at position C5 or C6 may not interfere with the redox activity of the fluorescent labelled ascorbic acid and therefore, may be considered as an effective indicator for oxidative stress by means of ESR and fluorescence spectroscopic studies. However, acylation at 5-CH-OH may prove to be disadvantageous, as a consequence of steric hindrance at this position. Thus, a newly synthesized fluorescent-labelled ascorbic acid from fraction 3 acylated at position 6 C-OH can be effectively used for further studies.

3.4.4 Mass spectroscopy of fractions 2 and 3

Determination of molecular weights of fractions 2 and 3 using mass spectroscopic techniques can be very helpful for the characterization of fluorescent-labelled ascorbic acid and to provide information on the mode of acylation. By ionization of fraction 2 or 3, the molecular

ions (M$^+$ ion) had identical peaks at m/z 309 in their mass spectra. The complete mass spectra for both the fractions are given in **Figures 72** and **73**.
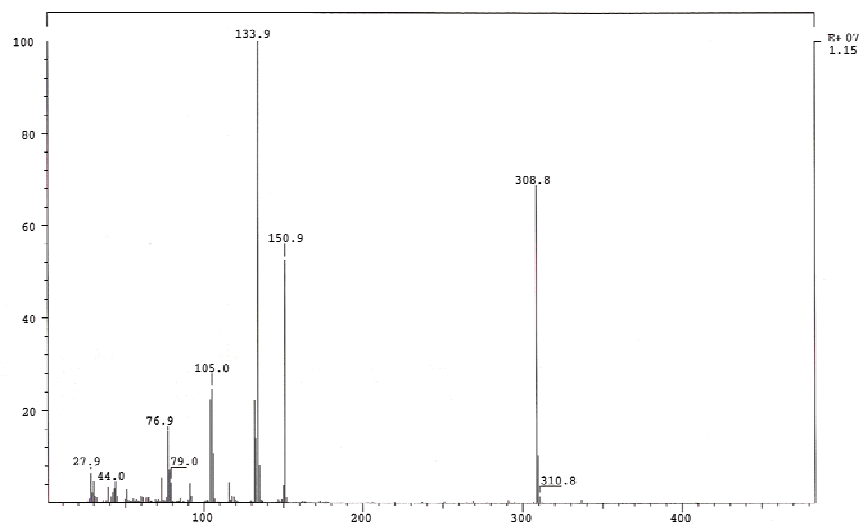
Figure 72 *Mass-spectroscopy: The fraction 2 showed peaks at m/z 308.8 in their mass spectrum.*

The plausible fragmentation process for fluorescent-labelled ascorbic acid of fraction 3 (**Figure 73**) involves the loss of carbon dioxide and carbon monoxide from the lactone ring to give the peaks at m/z 44, m/z 28 and the fragmentation of ascorbic acid (m/z 265). However, fragmentation can also occur at the attachment points to the aromatic ring producing elements of methylamino phenyl (m/z 106), phenyl cation (m/z 77), and methylamine (m/z 30). Another important fragmentation pattern involves α- and β-cleavage relative to the carbonyl group of ester to give the N-methylamino benzoyl (m/z = 134) and methylamino benzoxyl (m/z 150).

However, ionization of the fluorescent-labelled ascorbic acid ester of fraction 2 (**Figure 72**) showed almost similar fragmentation patterns, but with changes in their intensity ratios and fragmentations void between m/z 151 and 309. The most apparent fragmentation process was the loss of N-methlamino benzyl (m/z = 134) and to a lesser extend the loss of N-methylamino benzoxyl (m/z = 150). Moreover, molecular ion M$^+$ of fraction 2 showed a strong peak at m/z 309, which was the mass of the original compound.

Therefore, the information obtained from MS spectra confirms the molecular weight of both the fractions. From the fragmentation pattern, it was possible to assign the distinct molecular ion peaks of aromatic derivatives from the cleavage of the carbonyl groups of the esters. Furthermore, elements of carbon monoxide and carbon dioxide could also be traced from the MS spectra. These results confirm the modification of ascorbic acid with fluorescent label in fractions 2 and 3 acylated at two separate positions.
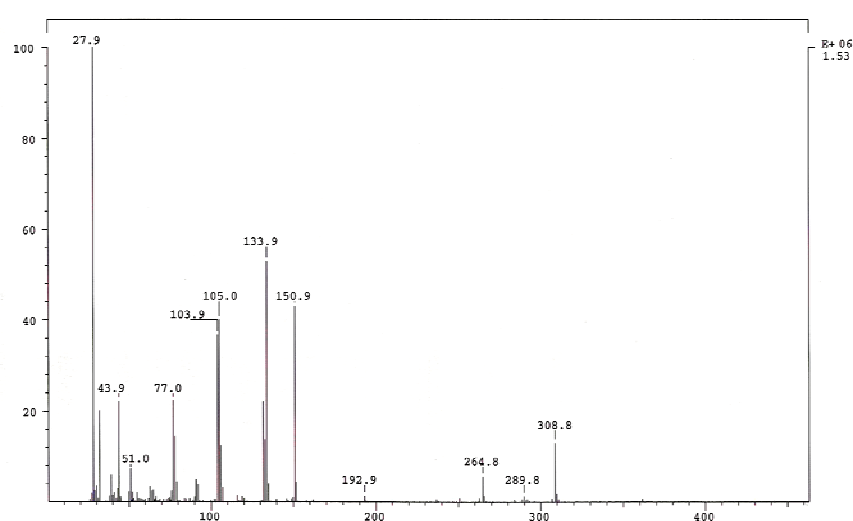
Figure 73 *Mass spectroscopy: The fraction 3 showed peaks at m/z 308.8 in their mass spectrum.*

Acylation of ascorbic acid with N-methylisatoic anhydride occurred mainly at position 3-C (fraction 2) on the ene-diol and position 6-C (fraction 3) on the side chain. For further studies only fraction 3 was considered because the acylation at position 6-C may probably not interfere with the antioxidant property of ascorbic acid. The redox activity occurs at the ene-diol group. Therefore further information on the chemistry of fraction 3, 6-O-(N-methylanthraniloyl) ascorbic acid or MANTA, is required to study its antioxidant capability.

3.4.5   UV spectroscopy of 6-O-(N-methylanthraniloyl) ascorbic acid (MANTA)

To assess the dynamics and stability of MANTA under various conditions, this new product was compared with educts and studied by UV spectroscopy. Superimposed spectra of ascorbate, N-metylisatoic anhydride and MANTA dissolved in 10 % acetonitrile/ PBS buffer

at pH 7.4 are characterized by their specific absorption bands as shown in **Figure 74**. Ascorbate showed a strong absorption band at 265 nm and N-methylisatoic anhydride at 250 nm and 330 nm respectively. Whereas the product MANTA showed at least 2 absorption peaks at 256 and 350 nm with an additional hump at 268 nm. Therefore, MANTA can be characterized by its specific absorptions band in reference to ascorbic acid and N-methylisatoic anhydride.
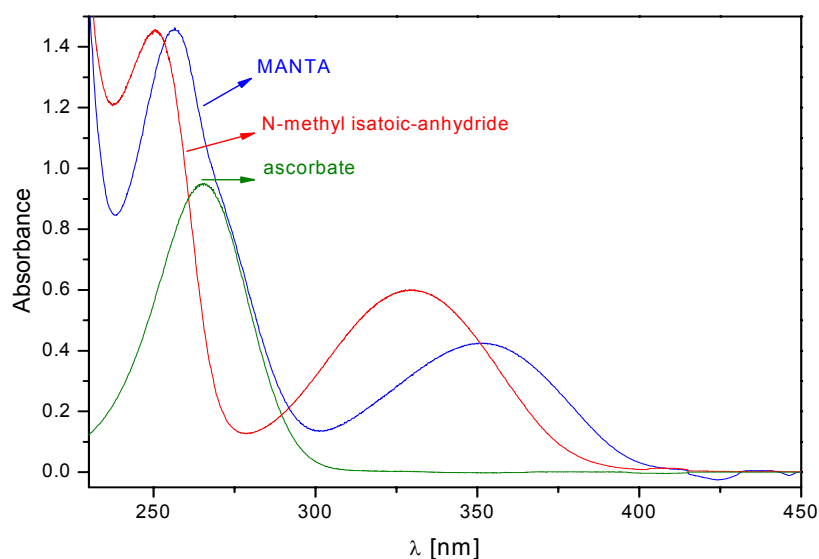
Figure 74 *UV spectra of ascorbate (——), N-methylisatoic anhydride (——) and MANTA (——) in PBS at pH 7.4 dissolved in 10 % acetonitrile as solvent.*

As discussed in the previous chapter, the chemistry of ascorbate oxidation vastly dependents on pH, concentration and oxidants present in a system. Therefore, it is possible to observe similar characteristics with MANTA exposed to acidic and weak basic medium. Firstly, absorbance of MANTA was investigated using UV spectroscopy at pH 4.6 and pH 7.2 respectively. Results obtained from UV spectra reveal a 33 % decrease in acidic environment as compared to the basic medium at the same concentrations (**Figure 75**). UV studies of MANTA clearly show the pH dependence of this newly synthesized molecule.
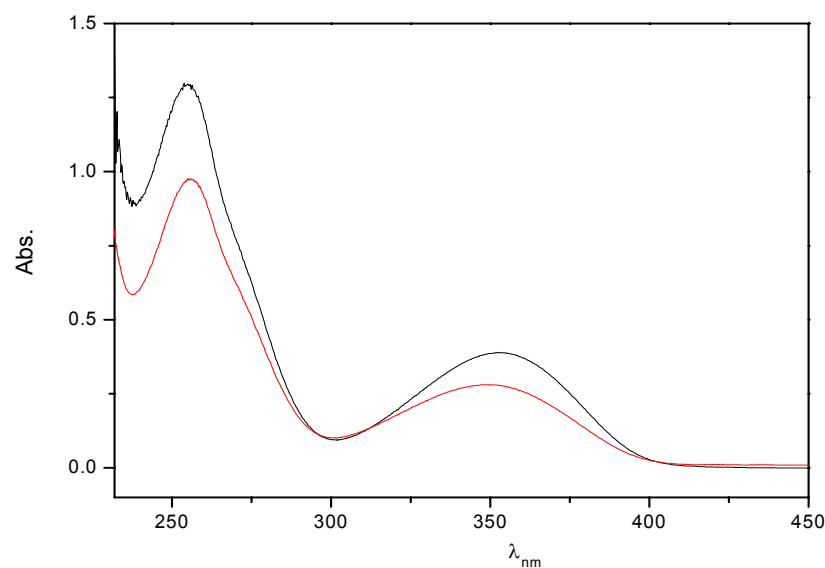
Figure 75 *UV spectra of the MANTA at pH 4.6(—) in sodium PCB medium and at pH 7.2(—) in PBS medium dissolved in 10 % acetonitrile. Absorbance maximum observed at 256 nm and 350 nm with a barely visible shoulder at 268 nm resulting from the absorption band of ascorbate.*

3.4.6 Fluorescence and ESR studies on redox reactions of fluorescence labelled ascorbic acid ester (MANTA)

Intramolecular quenching between covalently linked fluorophore quencher (paramagnetic molecules) pairs can be considered as an important method for the determination of free radicals in biological systems. During periods of oxidative stress, ROS occurring at hot spots in a radius of barely 20 Å are released to the surroundings. Subsequently, antioxidant capacity is only effective at these regions making it difficult to determine and quantify ROS in a living organism. Here, the newly synthesized fluorescent label may serve as a water-soluble antioxidant showing multifaceted characteristics with its involvement in biological systems. The dynamic redox chemistry of ascorbic acid makes it one of the most effective water-soluble antioxidant in living systems. Therefore, labelling it with a fluorescent marker may further help with investigations on oxidative stress. In addition, the involvement of ascorbate in intra and extra cellular reactions and regeneration can be followed efficiently with fluorescence.

Here, confocal laser fluorescence spectroscopic techniques may prove to be very valuable in localizing hot spots in plasma membrane redox systems. To assess the chemistry of MANTA in relation to its antioxidant capacity, several studies in the presence of ROS were carried out using fluorescence and ESR spectroscopy.
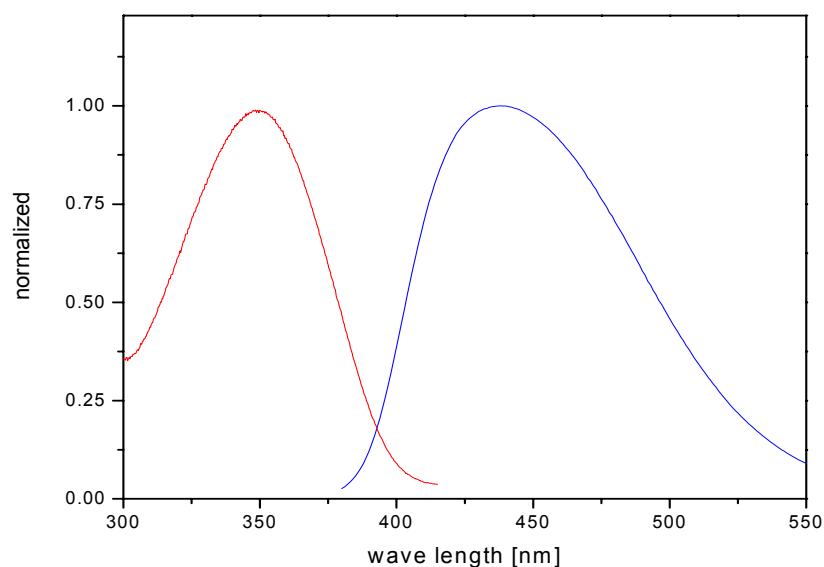
Figure 76 *Excitation (λ_{exc} = 350 nm(——)) and emission (λ_{em} = 446 nm(——)) spectra of MANTA in PBS at pH 7.4 in 10 % acetonitrile. UV and fluorescence spectra were normalized to fit absorbance and emissions scale.*

From the results obtained so far, further investigations were required to study fluorescence quenching and ascorbyl free radical generation of MANTA in phosphate buffers. Excitation and emission spectra of MANTA showed absorptions band at 350 nm and 446 nm respectively in 10% acetonitrile and PBS at pH 7.4 (**Figure 76**). To study the dynamics of MANTA in correlation with fluorescence quenching and MANTA free radical generation, chemical and enzymatic oxidants as source of ROS were incubated with this system. Formation of ROS was accomplished by adding 80 µM Fenton's reagent to 2.5 mM MANTA and the time-dependent decrease in fluorescence intensity was studied (**Figure 77**). Oxidation of MANTA induced changes in the fluorescence intensity resulting in a 10-fold decrease. This effect is likely attributed to fluorescence quenching as a consequence of a paramagnetic compound in the vicinity of a fluorescent molecule. Similarly, an ESR study on the oxidation of MANTA was carried out by adding 10 mM Fenton's reagent. A typical ESR signal was seen, consisting of a doublet with a hyperfine splitting a^{H4} = 1.8 G, which confirms the presence of the ascorbyl free radical derivative (**Figure 78**).
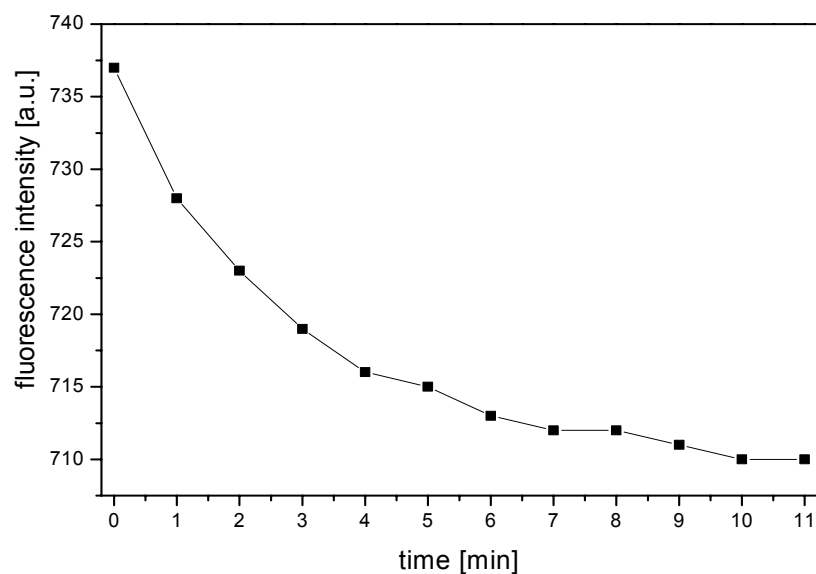
Figure 77 *Fluorescence quenching (−■−) of MANTA at λ_{emm} = 446 nm against time in 10 % acetonitrile after oxidation with 80 μM Fenton's reagent in PBS medium at pH 7.4.*
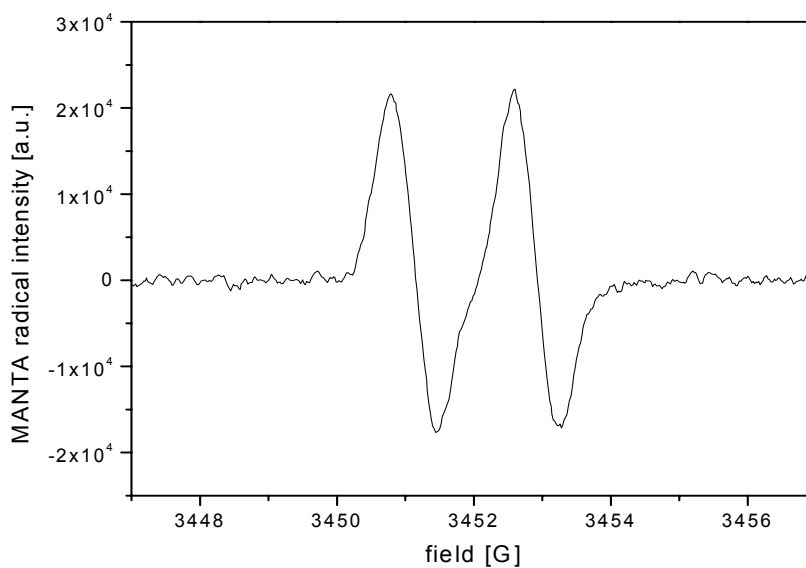
Figure 78 *ESR spectrum of product 6-O-(N-methylanthraniloyl) ascorbic acid or MANTA in PBS medium after oxidation with 10 mM Fenton's reagent.*

These results suggest that ESR and fluorescent studies on MANTA were successful. Fluorescence quenching and ascorbate oxidation could be established in reactions involving oxygen free radicals that showed the presence of a double labelled paramagnetic fluorescent

molecule at very close proximity. Therefore, further studies are able to show the redox-chemistry of MANTA as a function of pH as observed in the previous chapter on the chemistry of ascorbate. For ESR investigations, MANTA was oxidized with 1 mM Fenton's reagent as source of oxygen free radicals with a wide range of pH dependent buffers. Under the same experimental setup, fluorescence studies were also conducted with a 10-fold lower concentration of Fenton's reagent.
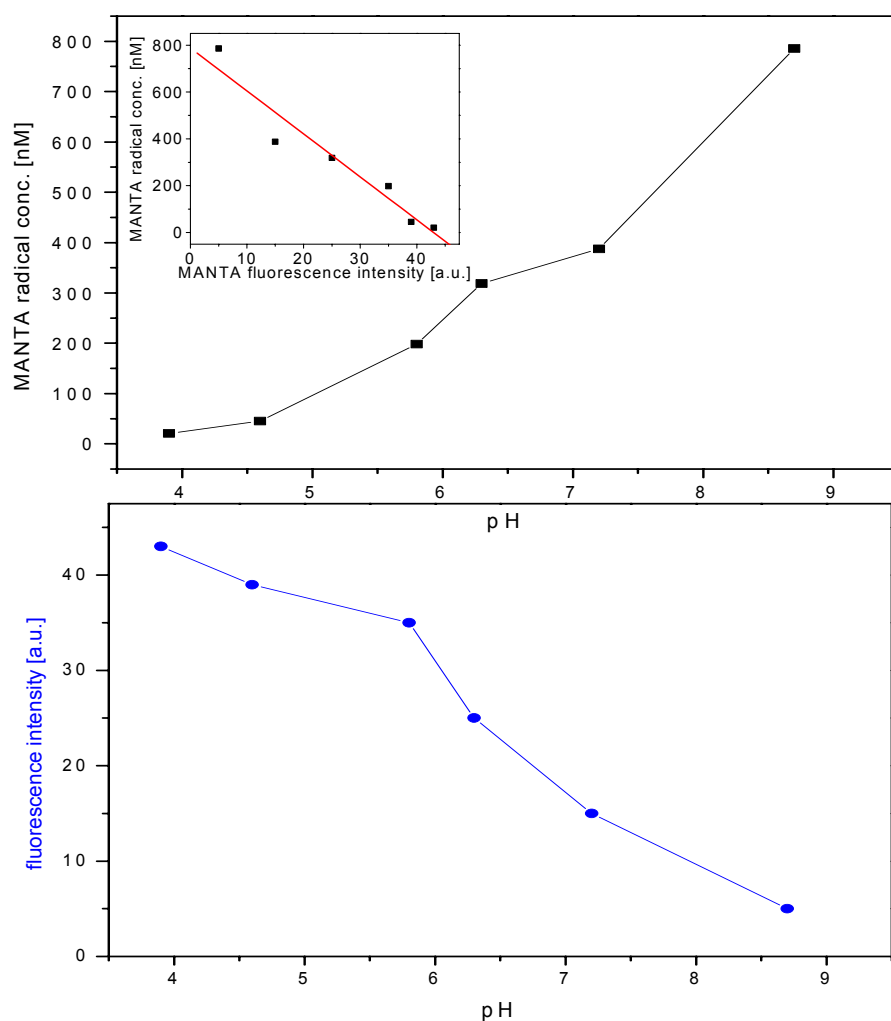
Figure 79 *Oxidation of MANTA in Fe^{2+}/ H_2O_2 observed under fluorescence (−■−) and ESR spectroscopy (−●−) against pH. Inset: negative correlations regression between fluorescence intensity, and radical concentration of MANTA.*

The data in **Figure 76** showed that the MANTA radical concentrations increased as a function of pH, whereas the fluorescent intensity decreased continually. Therefore, pH-dependent

cross-correlation pathways could be established within the same molecule. Based on the collected data, oxidation of MANTA revealed a strong pH dependency in the same way as seen with ascorbate in the previous chapter.

3.4.7 *in vitro* **ESR, fluorescence and UV spectroscopic studies on the oxidation of fluorescence-labelled ascorbic acid (MANTA) using the hypoxanthine and xanthine oxidase (XOD) system**

As described in the previous **section 3.3.2**, decomposition of purines to hypoxanthine or xanthine and finally to uric acid resulted in the release of superoxide radical anion as intermediate. As for ROS generating systems, hypoxanthine/xanthine oxidase assays were found to be an ideal choice for studying MANTA oxidations. Moreover, correlation studies using ESR and fluorescence spectroscopy can be applied to monitor time-dependent changes during oxidation. The results from these studies using fluorescence-labelled ascorbic acid may turn out to be appealing in the near future by observing hot spots in cell lines during periods of oxidative stress.
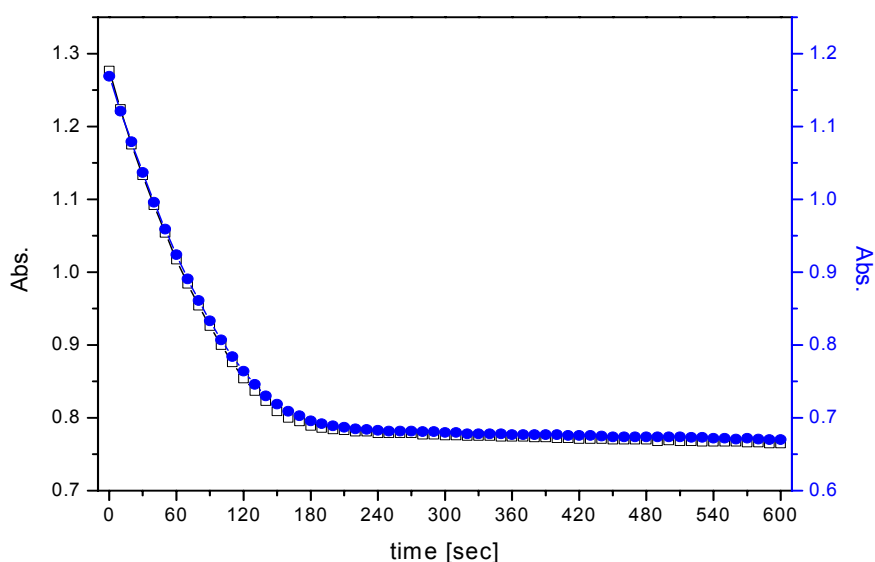
Figure 80 *Kinetics of 1 mM hypoxanthine oxidation after addition of 1 mg/ ml XOD in 5% DMF (–•–) and acetonitrile (–□–), measured using UV spectroscopy at 252 nm.*

To assess the role of MANTA as an antioxidant, hypoxanthine/ xanthine oxidase enzymatic reactions were utilized as source of oxygen free radicals and monitored using UV, ESR and fluorescence spectroscopic methods. Firstly, time-resolved hypoxanthine decomposition in xanthine oxidase complex incubated in PBS at pH 7.4 was monitored by UV spectroscopy at 252 nm. The data in **Figure 80** demonstrates the behaviour of hypoxanthine oxidation to uric acid in DMF and acetonitrile. Apparently, the kinetic rates were hardly affected after adding 5% of the above solvents to the reaction processes.
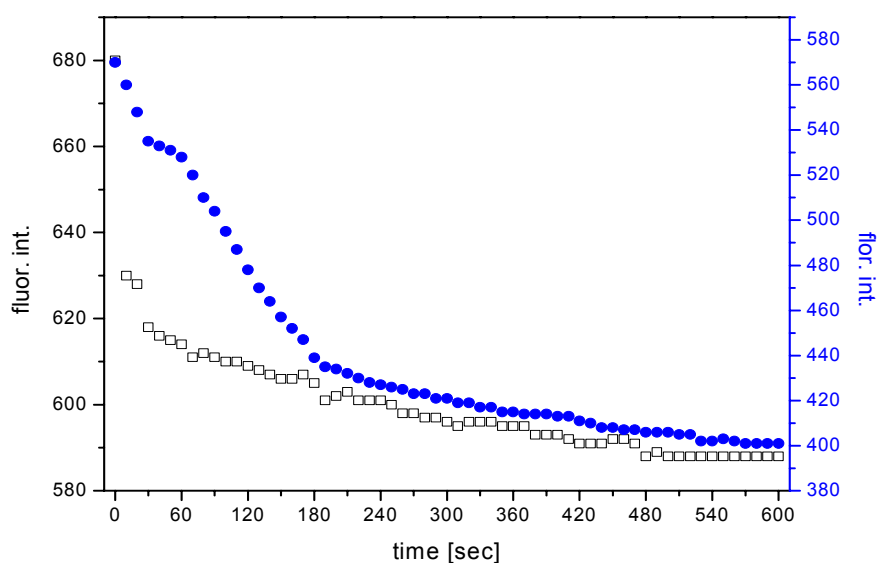
Figure 81 *The fluorescence quenching of MANTA at λ_{em}= 446 nm in 5% DMF (•) and acetonitrile (□) after oxidation with hypoxanthine/ xanthine oxidase.*

Under similar experimental setup as mentioned above, addition of hypoxanthine/ XOD in 3.3 mM MANTA induced a time-dependent decrease in fluorescence intensity at 446. Apparently, solvents like acetonitrile and DMF did not significantly affect fluorescence signal intensities. Nevertheless, some changes were observed during the first 3 minutes (**Figure 81**). To determine correlations between hypoxanthine oxidation and fluorescence quenching of MANTA, their kinetic data were superimposed and compared as seen in **Figures 82** and **83**.
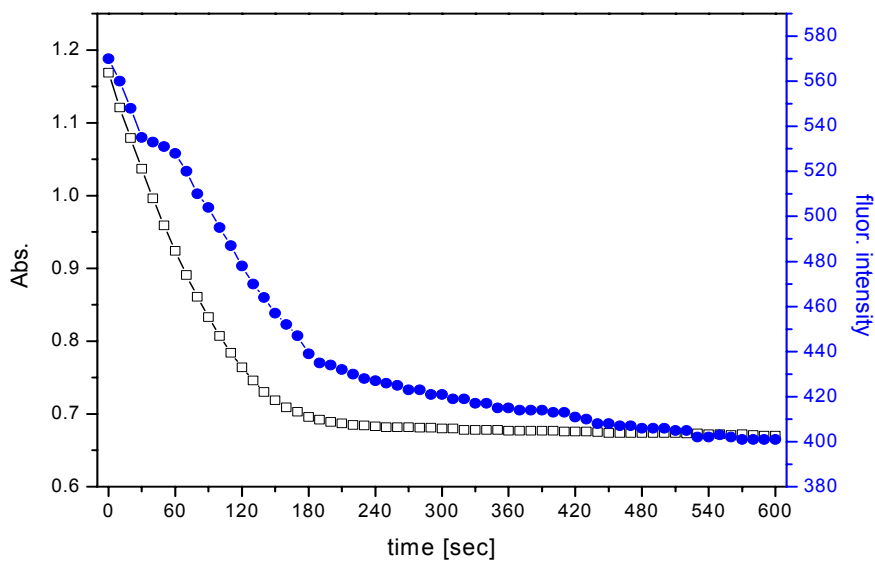
Figure 82 *Time-dependent hypoxanthine oxidation at λ_{exc} = 252 nm (–□–) in relation to fluorescence quenching of fraction 3 in 5 % DMF detected using fluorescence spectroscopy at λ_{exc} = 446 nm (–•–).*
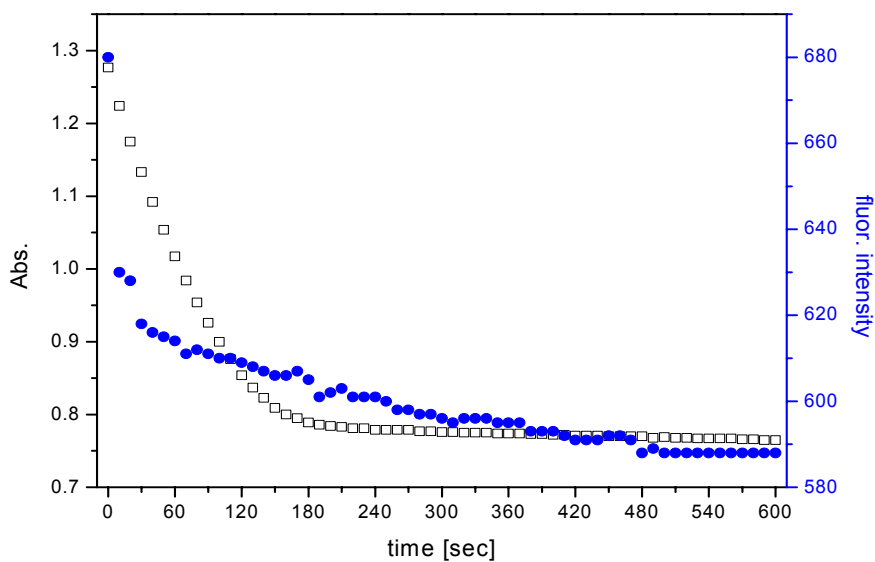
Figure 83 *Time-dependent hypoxanthine oxidation at λ_{exc} = 252 nm(□) measured under UV spectroscopy in relation to fluorescence quenching from fraction 3 in 5 % acetonitrile detected using fluorescence spectroscopy at λ_{exc} = 446 nm (•).*

Figures 82 and **83** showed good correlations between substrate decomposition using UV spectroscopy and MANTA oxidation observed by fluorescence spectroscopy. The results suggest a close relationship between hypoxanthine decomposition and MANTA oxidation as a consequence of superoxide radical anion intermediates. The experiments also showed that the enzymatic reactions were not affected by organic solvents. To access the possible relationship between fluorescence intensity and oxidation of MANTA, time-dependence of this fluorescent-labelled ascorbic acid ester was observed using ESR spectroscopy. An increase in the MANTA free radical concentrations was seen for the first 3 minutes followed by a decrease in radical signal intensity (**Figure 84**).
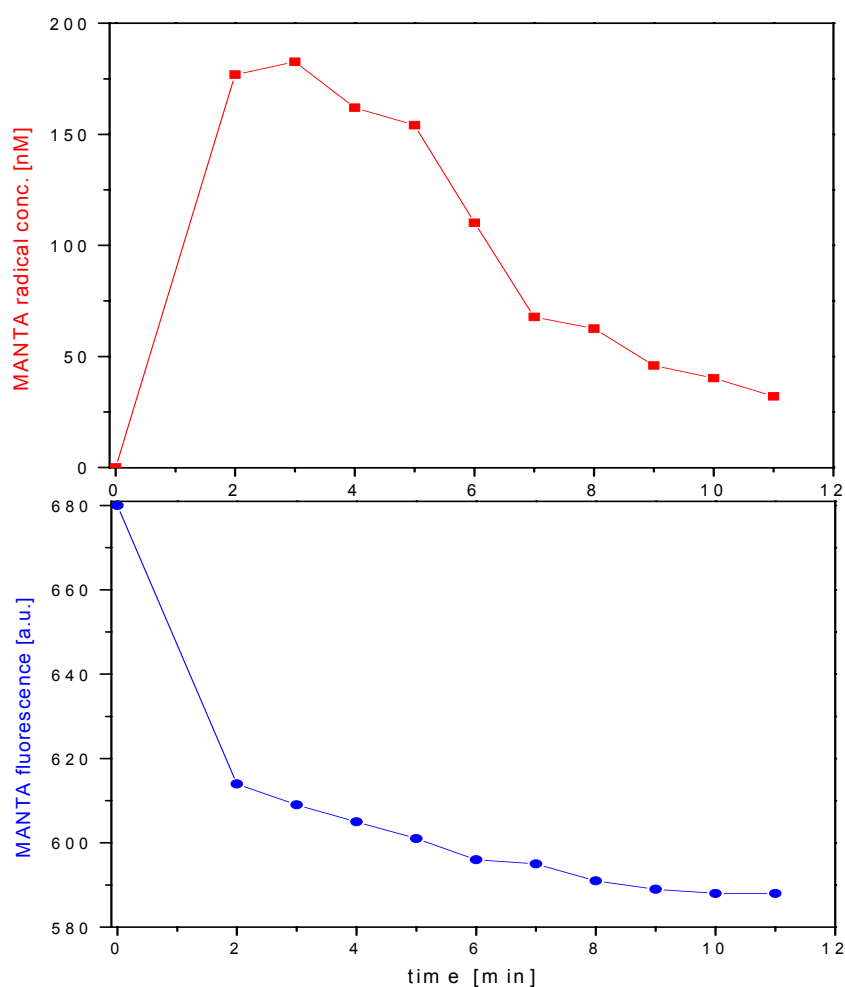
Figure 84 *Time-dependent oxidation of MANTA in a hypoxanthine/xanthine oxidase system. Correlations observed between the radical concentrations (–■–) and fluorescence quenching (–•–) of MANTA.*

Therefore, MANTA oxidation in radical environment followed a time-dependent pattern similarly to that of ascorbate oxidation as seen in **Figure 46**. Fluorescent intensity of MANTA on the other hand decreased continuously with time and remained stable after 10 minutes (**Figure 84**).

The data in **Figure 84** showed a cross-correlation for the first 3 minutes as a result of MANTA free radical oxidation and fluorescence quenching. Nevertheless, after 3 minutes of MANTA oxidation, fluorescence intensity dropped still further in relation to MANTA radical concentrations. A plausible explanation for the subsequent decrease in fluorescent intensity is the oxidation of fluorescent-labelled ascorbyl radical to dehydroascorbate. Apparently, dehydro MANTA can induce quenching similar to studies observed with quinone derivatives, that act as effective quenchers.

The results in this study provide evidence that a new fluorescent-labelled ascorbic acid ester (MANTA) was successfully synthesized from N-methyl isatoic anhydride and ascorbic acid. The multifaceted character of MANTA showing antioxidant properties was established using UV, ESR and fluorescence spectroscopy. MANTA is relatively stable in 5 % organic solvents like acetonitrile and DMF, unstable in DMSO and partly soluble in water. This fluorescent-labelled ascorbic acid ester was isolated by HPLC, purified and characterized using ^{1}H-NMR and mass spectroscopic methods. As for the aspects of MANTA oxidation with the help of fluorescence and ESR spectroscopy, time-dependent studies on fluorescence intensity and radical concentrations revealed unique structural and dynamic information. MANTA showed that it's modified structure, and redox properties towards oxidants do not differ from those of ascorbate. Particularly, both the compounds demonstrated similar properties such as pH dependency, type of ESR structural information of radical species and time-dependent oxidation kinetics. MANTA could also be easily oxidized by chemical and enzymatic reactions as source of oxygen free radicals. Another unique feature of MANTA is the emission of an intense blue colour at 446 nm, which could be useful in the future for real-time detection in cells using fluorescence confocal laser microscopic techniques. Moreover ROS can be easily targeted by observing areas of fluorescence quenching. Therefore by the application of ESR and fluorescence spectroscopic methods for targeting and determining ROS, MANTA probe may enhance and open new pathways for studies on oxidative stress responses in biological systems. Nevertheless, limitations of MANTA in the detection of ROS lie in its poor stability in aqueous medium. MANTA was relatively stable in reaction medium containing more than 5% organic solvents, but very unstable in DMSO. Therefore, studies on

the antioxidant activity of MANTA in organic solvents may turn out to be inadequate in biological systems in response to cell viability.

A possible reason for its instability could be the highly strained lactone ring caused by the acylation on 6 C-OH side chain of MANTA. Adding carbon spacers between the ascorbic acid and the fluorophore might solve this problem.
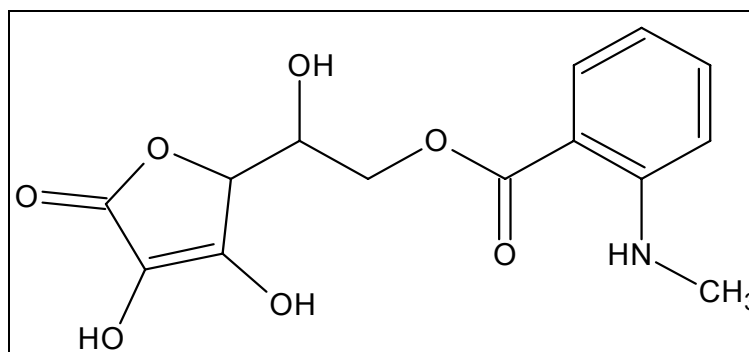
Figure 85 *Proposed structure and name of fluorescence labelled ascorbic acid ester of fraction 3: 6-O-(N-methylanthranilate) ascorbic acid or 2-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl)-2-hydroxyethyl 2-(methylamino)benzoate. Molecular formula, $C_{14}H_{15}NO_7$ and molecular weight, 309.271.*

3.5 Validation of routine ESR spectrometers with respect to the determination of ascorbyl radical concentration in plasma samples in comparism with the Bruker ELEXSYS E500 Series spectrometer (E580)

3.5.1 Ascorbyl free radical as a marker of oxidative stress: Quantitative analysis with e-Scan and E580 ESR spectrometers

In the course of this thesis a Bruker high end research ESR spectrometer (E 500 Series) was employed for the determination and quantification of ascorbyl free radical concentrations in plasma. The costs of such an instrument are certainly prohibitive for routine clinical applications, *i.e.*, routine determination of the radical. Hence, two low cost bench top spectrometers were tested in this respect: The MiniScope MS 200 from MagnetTech and the *e-scan* from Bruker Biospin. The results for the former have already been submitted in a report for Pascoe Pharmazeutische Präparate GmbH, the company sponsoring the clinical trial of high dose vitamin C infusion in patients undergoing cardiac bypass surgery. In the following, corresponding data for the *e-scan* spectrometer are described.

3.5.2 *e-scan* Spectrometer

The *e-scan* has initially been designed for food irradiation control and the determination of radicals formed in beer during shelf life. It is a desktop X-band ESR spectrometer optimised for measurements around g = 2 with a fully automated tuning bridge and thus, significantly reduced tuning time. Moreover, it can be equipped with an automatic sample changer. Probes enter the cavity rather conveniently by means of a capillary tube of 0.6 mm in diameter. If used manually, samples as well as washing solutions can *e.g.,* be sucked into the cavity by means of a water aspirator. The minimum volume required for reproducible signal intensities is 27 μl.

For the quantitative determination of ascorbyl radicals in plasma, two methods for spectral quantification were considered. With one method, spectral analysis was accomplished by reading the signal heights in relation to stable nitroxide standards using the integrated software. The other method was double integration of the ascorbyl radical signal by either exporting the data to the Origin plot program or directly with the integrated Bruker software. **Figures 86** and **87** show typical raw ESR spectra taken either with the the *e-scan* or E 580 spectrometer, respectively.
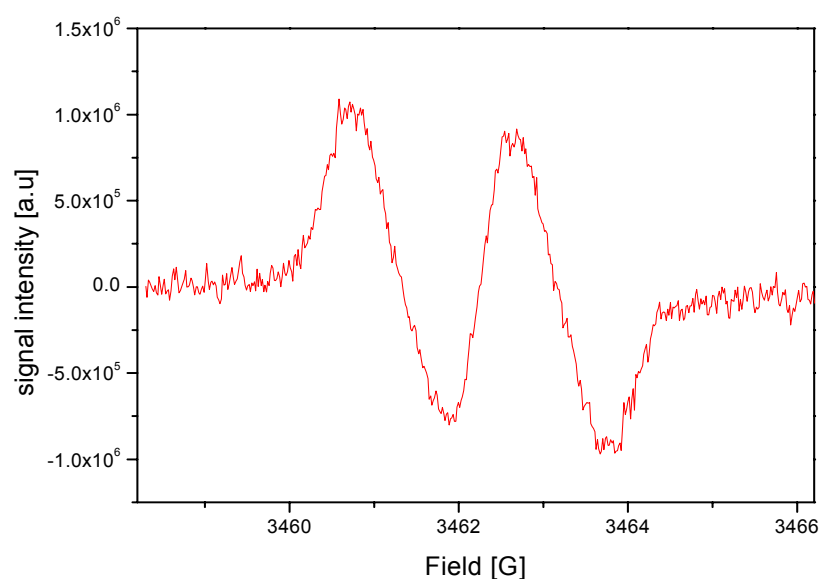
Figure 86 *Raw ESR spectrum of the ascorbyl radical, with a concentration of 137 nM, in human blood plasma recorded with the e-scan spectrometer.*

3.5.3   ELEXSYS E 580 ESR spectrometer

The ELEXSYS E 580, Fourier Transform (FT)-ESR spectrometer of the E 500 series is a front end research spectrometer with signal to noise (S/N) ratios up to 5000 and field accuracy of 500 mG over the full range. The blood plasma probes were inserted in a 1.1 mm quartz capillary requiring a minimum volume of 20 µl. Figure 80 illustrates the centre of maximum signal intensity for such a probe placed in the dielectric cavity that required a length of 4.5 cm. However, for routine measurements a quartz capillary was used with a length of 5.5 cm below the lower opening of the spacer from the sample rod and filled with 50 µl plasma sample.
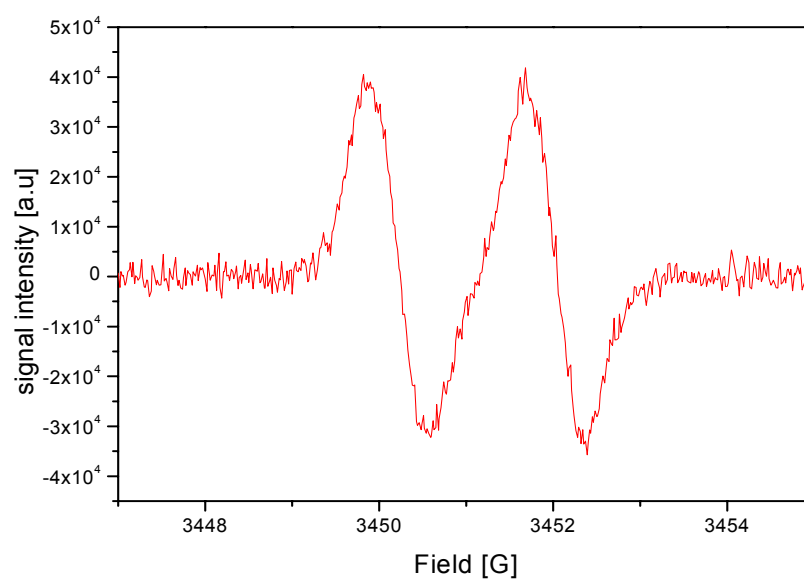
Figure 87 *ESR spectrum of the ascorbyl radical, with a concentration of 114 nM, in human blood plasma recorded with the E 580 spectrometer.*

ascr. conc. levels [nM]	double measurements of plasma probes	e-scan ascr conc. [nM]	E580 ascr conc. [nM]	Factor
≤ 100	32	36 ± 16	31 ± 13	**1.15 ± 0.18**
100 ≤ c ≤ 300	8	243 ± 25	216 ± 37	**1.20 ± 0.2**
≥ 300	**13**	**406 ± 112**	**336 ± 64**	**1.20 ± 0.19**

Table 1 *Quantification and comparison of ascorbyl radicals in human blood plasma, measured with the e scan and E 580 spectrometer. The spectral area of ascorbyl radicals was calculated with the Origin plot software.*

ascr. conc. levels [nM]	double measurements of plasma probes	Microcal Origin ascr conc. [nM]	Bruker ascr conc. [nM]	Factor
≤ 100	3	50 ± 7	49 ± 6	1.03 ± 0.00
100 ≤ c ≤ 300	4	240 ± 73	232 ± 71	1.03 ± 0.00
≥ 300	4	366 ± 15	354 ± 15	1.03 ± 0.00

Table 2 *Ascorbyl radical concentrations measured with the E 580 spectrometer. Concentrations were determined from the spectral area using integrated or the Origin plot software.*
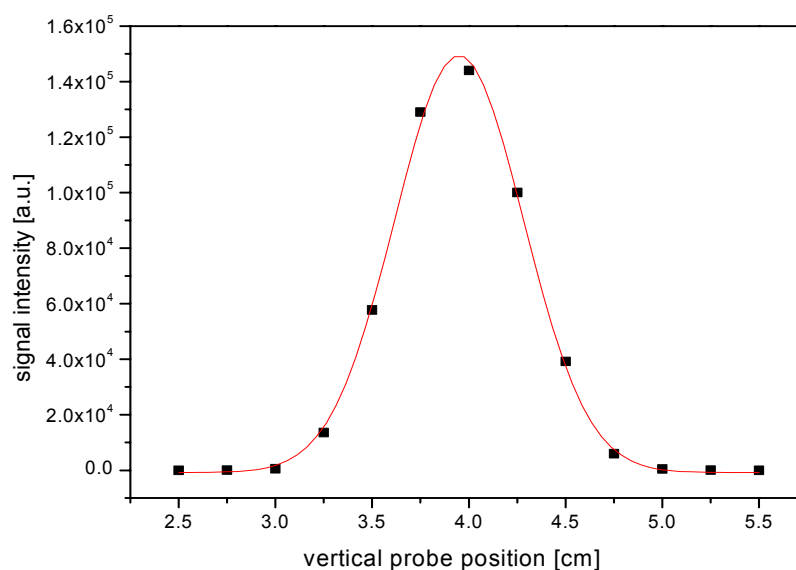
Figure 88 *Dependence of the signal intensity upon the vertical position of the probe in the Bruker dielectric cavity as determined with the E 580 spectrometer. Measurements were carried out using few TEMP-COOH nitroxide crystals fixed in silicone grease as point sample.*

ascr. conc. levels [nM]	no. of measurments	Quant: area ascr conc. [nM]	Quant: signal intensity ascr conc. [nM]	Factor
≤ 100	3	36 ± 16	136 ± 87	3.58 ± 0.79
100 ≤ c ≤ 300	4	167 ± 73	638 ± 373	3.81 ± 0.14
≥ 300	3	402 ± 99	1485 ± 402	3.68 ± 0.09

Table 3 *Quantification of ascorbyl radicals achieved by integration of spectral areas in relation to values derived from the signal intensities using an e-scan spectrometer.*

As can be seen in **Table 1**, the radical concentrations determined with the *e-scan* spectrometer are consistently higher by about 20 % as compared to the values determined with the E 580. At first look, this is rather surprising in consideration of the high sensitivity of the research instrument. However, as described in **Chapter 3.1.4**, the stability of the ascorbyl radical at room temperature is rather low, *i.e.,* spectra need to be recorded within 10 min at latest after thawing in order to avoid considerable decreases in signal amplitude. Apparently, the rapid sampling through the tubing system is not only compensating for the lower sensitivity of the routine instrument but even allows for better detection of the initial radical concentration.

Quantification by double integration is the method of choice and there are no differences whether the integrated software or the Origin program is employed (**Table 2**). Obviously, values based instead on the signal amplitude are highly in error (**Table 3**), most likely because of different line widths and shapes of the ascorbyl radical in relation to the nitroxides used as a standard in these experiments.

3.5.4 Instrument settings for the *e-scan* and E 580 spectrometers

e-scan		E580	
Resonator	: scc_0143.cal	Resonator	: diel.
Microwave frequency	: 9.74 GHz	Microwave frequency	: 9.69 GHz
Microwave power	: 7.06 mW	Microwave power	: 20.17 mW
Receiver gain	: 3.17e+03	Receiver gain	: 85
Mod. frequency	: 86 KHz	Mod. frequency	: 100 KHz
Mod. amplitude	: 0.99 G	Mod. amplitude	: 1 G
No. of scans	: 80	No. of scans	: 5
Center field	: 3462 G	Center field	: 3451 G
Sweep width	: 8 G	Sweep width	: 8 G
Resolution	: 512 points	Resolution	: 512 points
Conversion time	: 10.24 ms	Conversion time	: 163,84 ms
Time constant	: 10.24 ms	Time constant	: 81.9 ms
Sweep time	: 5.24 s	Sweep time	: 80 s

4 Material and methods

4.1 Biochemical studies of the efficacy of heavy-dose vitamin C supplementation on healthy subjects

4.1.1 Protocol of healthy subjects with intravenous infusion of 7.5 g and/ or 750 mg vitamin C

Number of male healthy volunteers	: 6
Intravenous infusion of Vitamin C-Injektopas$^{®}$	: 7.5 g and 750 mg
Time taken for intravenous	: 30 min/day in the morning
Duration of vitamin C infusion	: 6 days
Period under observation	: 29 days
Washout phase	: 14 days
Withdrawals of blood on following days	: 0 and 22/30 ml blood, once
	1 and 23/160 ml blood, 8 times
	2 to 6 and 24 to 28/50 ml blood, 5 times
Moreover urine probes taken at	: 0, 1, 22, 23 and 28 days respectively.

4.1.2 Vitamin C-Injektopas$^{®}$ dosage and clinical parameters

Division of healthy subjects	: 2 × 2 groups
Subdivision of healthy volunteers	: A1 and A2
Group A: day1	: 750 mg vitamin C intravenous
days 2-6	: 750 mg vitamin C intravenous
Group A subdivided into two groups:	
Group A1	: observation day 1
Group A2	: observation day 1-6

The procedure with 7.5 g vitamin C is repeated analogue experimental setup A

The vitamin C infusion with Vitamin C-Injektopas® was prepared and executed as follows:

1. Vitamin C-Injektopas® 750 mg: The infusion was administrated over a period of 10 minutes through a vena cubitalis diverted vein catheter.

2. Vitamin C-Injektopas® 7.5 g: Diluted in 50 ml flask with a 50 ml Ampuwa®. The infusion was passed over a period of 30 minutes through a vena cubitalis diverted vein catheter.

4.1.3 Withdrawal and storage of blood

Venous blood was drawn by venipuncture with an evacuated Vacutainer®. Blood tubes were drawn by 1 ml Lavender Top containing 15%Liquid K3 EDTA as anticoagulant. Sample tubes were transferred and centrifuged at 1500 X g for 5 minutes at 4° C. The non haemolysed plasma sample was decanted into 4×50 µl eppendorfs using transfer pipette and immediately shock frozen by liquid air. The samples were stored at -80° C or lower (transported on dry ice).

ascorbic acid	ascorbyl radical	Vitamin E
500 µl EDTA-plasma, meta-phosphoric acid, 2 * Eppendorfs, - 20°C	1 ml EDTA plasma, centrifuge, 4 * Epi´s, - 80°C	1ml EDTA plasma, 2 * Epi´s, - 20°C

Table 4 *Collection and storage of blood plasma.*

4.1.4 Separation and classification of biochemical parameters

Primary parameters	Secondary parameters
Ascorbic acid, ascorbyl radical conc. in plasma	MDA, 8-Oxoguanine, oxalat calcium

Table 5 *Classification of biochemical parameters into primary and secondary parameters.*

4.1.5 Primary Parameter: Determination of ascorbyl radicals in blood plasma of healthy subjects using ESR spectroscopy

Plasma probes frozen at -80°C were thawed immediately in 30°C water, vortexed shortly and inserted into a 1 mm quartz capillary tube with a 1 ml syringe. The probe was immediately inserted into the ESR cavity and measured at 25°C using a Bruker E 580-X band spectrometer operating at 9.4 GHz with a 100-kHz modulation frequency, equipped with a TE_{01} dielectric cavity. The concentrations of ascorbyl radical were determined by double integration of ESR spectra with TEMP-COOH as a standard. The nitroxide spectra of the standard were obtained with the same instrumental settings as used for ascorbyl radicals, except for the sweep width.

time [min]	0	5	15	30	60	120	240	480
7.5 g vit. C								
average ascr [nM]	47	137	132	219	130	107	77	56
stdev	13	89	57	103	52	27	19	15
750 mg vit. C								
average ascr [nM]	37	70	78	80	66	63	45	45
stdev	7	24	31	33	22	26	20	24

Table 6 *Time-dependent changes of ascorbyl radical concentrations from 6 healthy subjects after 7.5 g and/or 750 mg vitamin C infusion.*

Vit. C	750 mg	7.5 g	0
time [min]	ascr [nM]	ascr [nM]	ascr [nM]
12	151	265	30
30	145	225	29
45	123	175	28
60	94	152	29
75	85	120	25
90	69	98	22
105	61	75	9
120	46	49	15
135	40	39	9

150	27	26	10
165	25	18	9
180	23	14	--
195	17	14	--

Table 7 *Ascorbyl radical decay in plasma at laboratory conditions against time.*

4.1.6 Primary Parameter: Determination of ascorbic acid concentrations in blood plasma[*]

The refrozen plasma-EDTA-phosphoric acid mixture was added to 200 µl BHT-solution (buthylhydroxytoluene), vortexed gently for 1 min and centrifuged. 1:1 supernatant of the plasma mixture and moving phase (acetonitrile/ phosphate buffer at 70:30), once again centrifuged and injected through a GromSil 120 Amino-2pA 5 µM column at 12 mPa with a flow rate of 1.5 ml/ min.

time [min]	0	5	15	30	60	120	240	480
7.5g vit. C								
average ascOH [µM]	58	7061	2148	1837	1278	806	425	209
stdev	15	5202	280	288	164	160	116	59
750 mg vit. C								
average ascOH [µM]	73	1905	311	243	204	160	118	91
stdev	27	1508	93	76	29	47	28	30

Table 8 *Time-dependent changes of ascorbate concentrations of 6 healthy subjects in plasma after vitamin C infusion.*

*Silke Mrosek at the university of Hohenheim

4.1.7 Secondary parameter in plasma: MDA, vitamin E, 8-Oxoguanine, calcium, and oxalat levels in blood plasma (*determined at the university of Hohenheim*)

Protocol: MDA

Plasma TBARS mixture was incubated, heated and the complex eventually extracted with butanol. Measured under fluorescence spectroscopy: $\lambda_{exc} = 530$ nm and $\lambda_{em} = 590$ nm. The MDA concentrations obtained were proportional to lipid peroxidation equivalent levels.

Protocol: Vitamin E

For the determination of α-tocopherol in plasma, the plasma probes were added with ethanol for protein precipitation and extracted with n-hexane. The organic phase was directly injected into a Cyano 2PR HPLC column with n-hexane/isopropanol as moving phase and detected at $\lambda = 293$ nm. Quantifications were carried out with given standards of tocopherol concentrations

Protocol: 8-oxoguanine

8-OHdG and 8-OH-G excreted in urine was separated through a C18 ODS 2 column in neutral medium and once again separated with a nucleoside specific column and electrochemically detected in acid medium.

Protocol: Oxalate

For the determination of oxalate concentration, it was oxidized to carbon dioxide and hydrogen peroxide by oxalate-oxidase. Hydrogen peroxide in turn reacted with 3-methyl-2-benzothiazolium-hydrazone (MBTH) and 3-dimethylamino-benzoeic acid in the presence of peroxidase, in which an indamine-chromophore complex appeared with absorptions maximum of $\lambda = 590$ nm which is proportional to the oxalate concentration in the probe.

Sec. parameter in urine	Vit. C dosage	Day 0	Day 1	Day 6
vitamin E [μM]	750 mg	25.7 ± 4.3	26.2 ± 4.7	25.1 ± 7.3
	7.5 g	27.8 ± 8.5	26.9 ± 3.8	24.4 ± 4.7
MDA [μM]	750 mg	0.53 ± 0.13	0.46 ± 0.07	0.36 ± 0.03

	7.5 g	0.58 ± 0.15	0.43 ± 0.11	0.40 ± 0.08
8-oxoguanine	750 mg	35.9 + 35.2	19 + 19,5	29.9 + 14.6
	7.5 g	34.4 + 23.3	45,5 + 33	35.3 + 25.9
Oxalat	750 mg	27.3 + 9.2	30 + 6.3	35.2 + 5.1
	7.5 g	37.7+ 9.2	28.8 + 7.6	52.3 + 11.7
Calcium [mM]	750 mg	5.33 + 1.53	5.03 + 2.25	3.68 + 3.14
	7.5 g	5.53 + 1.54	5.42 + 1.59	2.55 + 1.53

Table 9 *Secondary parameters for oxidative stress in urine after vitamin C supplementation.*

4.2 Biochemical correlations of oxidative stress parameters from patients undergoing an aorta-coronary bypass operation after heavy doses vitamin C therapy

4.2.1 Classification of patients in treatment groups

group 1	placebo- patients
group 2	patients with bolus: 7.5g ASC und continuous infusion: 22.5g ASC
group 3	Patients with bolus: 15g ASC und continuous infusion: 30g ASC

Table 10 *Patients classified into 3 groups treated with placebo and vitamin C.*

	group 1	group 2	group 3	total
no. of patients	17	20	20	57

Table 11 *Total number of patients classified into 3 groups.*

4.2.2 Determination of ascorbic acid and ascorbyl radical, MDA, TAS, sICAM and sVCAM concentrations in patients after placebo, 30 g and 45 g vitamin C infusion.

4.2.2.1 Protocol : Ascorbyl radical determination using ESR spectroscopy

Frozen plasma probes were thawed immediately in 30°C water and measured at 25°C e with a Bruker E 580-X band spectrometer fitted with a TE_{01} dielectric cavity. The quantification of ascorbyl radicals was performed, by plotting the concentrations of a stable nitroxide radical (3-carboxyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl) as standard, against the area of ESR spectra after double integration. To fit the data a linear regression was applied. Ascorbyl radical concentrations were determined after double integration.

ascr [nM]	placebo	22.5 + 7.5 g	30 + 15 g
B1	34 ± 8	32 ± 14	48 ± 35
B2	22 ± 12	188 ± 58	237 ± 87
B3	33 ± 9	224 ± 67	280 ± 98
B4	34 ± 8	227 ± 64	271 ± 94
B5	36 ± 10	245 ± 71	284 ± 83
B6	37 ± 8	249 ± 70	302 ± 77
B7	29 ± 8	209 ± 48	279 ± 67
B8	29 ± 7	179 ± 48	247 ± 66
B9	27 ± 7	126 ± 43	173 ± 49
B10	27 ± 5	48 ± 12	46 ± 18

Table 12 *Ascorbyl radical concentrations after vitamin C infusion from placebo patients and patients after vitamin C infusion. B1 to B10 (see **chapter 3.2.1**)*

4.2.2.2 Protocol: Ascorbic acid determination*

The total ascorbic acid as well as dehydroascorbate was determined by a photometric method. Dilute *m*-phosphoric acid was added to 10 times the volume of EDTA-plasma and stored at -80°C. The plasma was thawed and oxidized with TEMPO to dehydroascorbate which in turn reacts with *o*-phenylenediamine to a coloured product with an absorptions maximum at λ_{max} = 340 nm.

ascOH [μM]	placebo	22.5 + 7.5 g	30 + 15 g
B1	45 + 12	48 + 41	51 + 9
B2	33 + 9	965 + 380	1875 + 611
B3	40 + 9	1197 + 472	2575 + 893
B4	41 + 10	1190 + 444	2420 + 841
B5	39 + 10	1232 + 504	2500 + 883
B6	36 + 10	1160 + 479	2406 + 994
B7	31 + 7	912 + 373	1845 + 769
B8	31 + 7	827 + 347	1579 + 832
B9	32 + 9	758 + 433	1386 + 855
B10	22 + 9	60 + 33	61 + 15

Table 13 *Ascorbate concentrations after vitamin C infusion from placebo patients and patients after vitamin C infusion.*

4.2.2.3 Protocol: Determination of MDA levels in plasma

See section **4.1.7**

MDA [μM]	placebo	22.5 + 7.5 g	30 + 15 g
B1	0.33 + 0.10	0.34 + 0.1	0.37 + 0.08
B2	0.30 + 0.08	0.21 + 0.05	0.26 + 0.05
B3	0.40 + 0.11	0.32 + 0.07	0.44 + 0.11
B4	0.40 + 0.11	0.33 + 0.07	0.42 + 0.11
B5	0.42 + 0.10	0.34 + 0.06	0.46 + 0.13
B6	0.52 + 0.17	0.39 + 0.10	0.55 + 0.22
B7	0.75 + 0.26	0.49 + 0.17	0.60 + 0.20
B8	0.80 + 0.34	0.48 + 0.19	0.51 + 0.10
B9	0.45 + 0.14	0.30 + 0.10	0.39 + 0.11
B10	0.48 + 0.18	0.35 + 0.09	0.37 + 0.09

Table 14 *MDA concentrations after vitamin C infusion from placebo patients and patients after vitamin C infusion.*

4.2.2.3 Protocol: TAS

The TAS was measured using a colorimetric assay. The chromogen ABTS is incubated with peroxidase and hydrogen peroxide to produce the ABTS radical cation. The ABTS radical is detectable from its blue-green colour which is measured at 600 nm at 37°C. Antioxidants in the sample suppress the formation of the radical cation to a degree which is proportional to their concentration.

TAS [mM]	placebo	22.5 + 7.5 g
B1	1.34 $\pm$ 0.14	1.35 $\pm$ 0.14
B2	1.06 $\pm$ 0.15	1.60 $\pm$ 0.06
B3	1.26 $\pm$ 0.18	1.61 $\pm$ 0.05
B4	1.26 $\pm$ 0.15	1.62 $\pm$ 0.05
B5	1.27 $\pm$ 0.16	1.61 $\pm$ 0.04
B6	1.32 $\pm$ 0.14	1.61 $\pm$ 0.04
B7	1.31 $\pm$ 0.15	1.61 $\pm$ 0.05
B8	1.32 $\pm$ 0.16	1.61 $\pm$ 0.06
B9	1.29 $\pm$ 0.14	1.60 $\pm$ 0.06
B10	1.34 $\pm$ 0.13	1.34 $\pm$ 0.17

Table 15 *Total antioxidant status after vitamin C infusion from placebo patients and patients after vitamin C infusion.*

* Vitamin C, TAS und TBARS: Marina Langer, Universität Hohenheim, Institut für Biologische Chemie und Ernährungswissenschaft

4.2.2.4 Protocol: sICAM, sVCAM*

A monoclonal antibody specific for sVCAM/sICAM was pre-coated into microplates. Standards, samples, controls and conjugates were pipetted into the wells and any sVCAM/ sICAM present was sandwiched between by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sVCAM. After washing a substrate solution (tetramethylbenzidine) was added into the wells. After 30 minutes the colour development was observed at 450 nm which is proportional to the amount of sVCAM/sICAM bound. The unknown concentration of sVCAM/sICAM in plasma was determined from the quantification of the standards.

ICAM [ng/ml]	placebo	22.5 + 7.5 g	30 + 15 g
B1	208 $\pm$ 57	196 $\pm$ 48	158 $\pm$ 31
B2	119 $\pm$ 36	113 $\pm$ 35	79 $\pm$ 31
B3	115 $\pm$ 31	115 $\pm$ 33	79 $\pm$ 14
B4	119 $\pm$ 33	112 $\pm$ 33	81 $\pm$ 14
B5	127 $\pm$ 36	121 $\pm$ 44	86 $\pm$ 19
B6	147 $\pm$ 44	135 $\pm$ 44	93 $\pm$ 19
B7	176 $\pm$ 50	159 $\pm$ 41	115 $\pm$ 10
B8	180 $\pm$ 61	163 $\pm$ 43	107 $\pm$ 11
B9	243 $\pm$ 75	193 $\pm$ 51	147 $\pm$ 36
B10	311 $\pm$ 113	246 $\pm$ 59	211 $\pm$ 29

Table 16 *sICAM levels after vitamin C infusion of placebo patients and patients after vitamin C infusion.*

VCAM [ng/ml]	placebo	22.5 + 7.5 g	30 + 15 g
B1	308 $\pm$ 136	296 $\pm$ 109	266 $\pm$ 110
B2	146 $\pm$ 59	152 $\pm$ 49	111 $\pm$ 34
B3	169 $\pm$ 96	159 $\pm$ 67	135 $\pm$ 38
B4	176 $\pm$ 78	171 $\pm$ 62	141 $\pm$ 46
B5	200 $\pm$ 81	182 $\pm$ 74	155 $\pm$ 38
B6	268 $\pm$ 121	240 $\pm$ 155	158 $\pm$ 75
B7	336 $\pm$ 121	325 $\pm$ 144	236 $\pm$ 30
B8	352 $\pm$ 184	293 $\pm$ 128	219 $\pm$ 53
B9	392 $\pm$ 261	318 $\pm$ 136	310 $\pm$ 69
B10	486 $\pm$ 264	421 $\pm$ 233	399 $\pm$ 164

Table 17 *sVCAM levels after vitamin C infusion from placebo patients and patients after vitamin C infusion.*

* VCAM und ICAM: Beate Schlegel, Universität Hohenheim, Institut für Biologische Chemie und Ernährungswissenschaft

4.3 Chemistry and detection of ascorbyl free radicals and spin trapping techniques *in vitro* as markers of oxidative stress

4.3.1 Dependence of ascorbyl radical concentration with varying pH's

Materials: hydrogen peroxide 30% Merck, ammoniumferrous (II) sulphate hexahydrate Merck, L(+) ascorbic acid, Riedel-de Haen.

Experiments were performed at 25°C by incubating 6 mM ascorbate concentration in pH dependent buffers with 0.6 mM hydrogen peroxide and 0.06 mM ammonium ferrous sulphate and eventually vortexed for 15-30 seconds. ESR spectra of ascorbyl radicals were obtained using an E580 spectrometer from Bruker operating at 9.4 GHz equipped with a TE_{01} dielectric cavity.

pH	conc [µM]
3.7	0.023 $\pm$ 0.02
4.4	0.069 $\pm$ 0.03
4.9	0.11 $\pm$ 0.02

5.6	0.154 ± 0.02
6.0	0.135 ± 0.03
6.4	0.291 ± 0.04
6.8	0.563 ± 0.05
7.0	0.612 ± 0.04
7.2	0.726 ± 0.07
7.6	0.932 ± 0.08
7.7	0.956 ± 0.1
8.1	2.188 ± 0.3
8.8	3.104 ± 0.6
9.4	1.919 ± 0.4
9.7	1.289 ± 0.6
9.8	0.938 ± 0.4

Table 18 *Ascorbyl radical concentrations in a wide range of pH Buffers.*

Ascorbyl radical concentrations were determined in pH dependent buffer mediums. Sodium phosphate-Citrate buffers were used pH's 3.6 to 5.4. At higher pH's 5.8 − 8.0 Sodium phosphate buffers were mixed whereas Borax buffers were used at pH above 8.0 to 10.

4.3.2 Oxidation of ascorbate in hypoxanthine and xanthine systems

Materials: Xanthine was obtained from Sigma Aldrich Germany and hypoxanthine was obtained from Fluka. Xanthine oxidase 10.7 mg/ml was purchased form Serva.

PBS 10X stock solution at pH 7.4

2.2 g	KH_2PO_4	20 mM
11.36	Na_2HPO_4	80 mM
1.86	KCl	25 mM
81.8 g	NaCl	1.4 M

Xanthine/ xanthine oxidase assay

1.07 mg/ ml xanthine oxidase catalyst in 10 mM xanthine substrate concentration was used as source of free radical generating system in 0.312 mM ascorbate medium in PBS at pH 7.4 and measured using ESR spectroscopy.

Xanthine

- 15.2 mg Xanthine
- 1 ml Na_2CO_3 solution 1 M

- add to 10 ml PBS 7.4

EDTA-Na$_2$

- 37 mg EDTA-Na$_2$
- add to 10 ml PBS 7.4

(NH$_4$)$_2$SO$_4$ solution

- 26. 4 g (NH$_4$)$_2$SO$_4$ solution 2M
- add to 100 ml H$_2$O (deionised)

Enzyme sample

- 180 µl (NH$_4$)$_2$SO$_4$ solution 2M
- 20 µl XOD stock solution

Substrate buffer solution

- 19.4 ml PBS buffer
- 0.4 ml xanthine suspension 10 mM
- 0.2 ml EDTA-Na$_2$ solution 10 mM in PBS

For UV spectroscopic measurements, 50 µl enzyme sample was added to 2.95 ml buffer substrate solution and measured at λ_{max} = 283 nm every 2 second for 2 minutes or otherwise specified.

Kinetics of ascorbyl radical concentrations in xanthine/ XOD systems after repeated introduction of XOD

Experimental procedure same as before, additionally 1.07 mg/ml xanthine oxidase was added after 20 min.

4.3.3   Spin trapping Methods using DMPO

Materials: DMPO was purchased from Sigma Aldrich, Germany.

Purification procedure for DMPO

1g commercial DMPO was purified by dissolving it in 5 ml de-ionised water. After addition of 5 ml toluene, the biphasic mixture were shaken vigorously and allowed to separate. The top toluene phase was pipetted out and discarded. This procedure was repeated twice before blowing in nitrogen to remove traces of toluene.

Meanwhile 1g of charcoal was stirred with 25 ml de-ionised water and filtered at the pump to remove traces of metal ions and eventually washed with 1L de-ionized water. The wet charcoal was added to a plastic centrifuge tube along with the toluene washed DMPO solution. The mixture was shaken vigorously for a minute or two and then centrifuged. The colourless DMPO was pipetted out and filtered through a syringe filter. The concentration of the resulting solution was determined by UV spectroscopy, λ_{max} = 227 nm, ε = 7700, which was about 1.5 mol DMPO. The stock solution was eventually stored at – 20°C.

In vitro determination of ROS was performed using 44 mM DMPO in 10mM Xanthine PBS medium. Before starting the reaction, 1.07 mg/ml xanthine oxidase was added and immediately measured with ESR with 100 G scan width.

4.3.4   Vitamin C and vitamin E interactions in biphasic systems

Materials: Tocopherol, Triton-X 100 from Sigma

1M α-Tocopherol was added to 10 % triton-X 100 and PBS buffer at pH 7.4 and vortexed with 10 mM H_2O_2 and 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ for 10 min. The tocopherol radicals were extracted with equal volume of ethyl acetate. Meanwhile, 10 mM of ascorbate was prepared in PBS medium at pH 7.4. The biphasic mixture was vortexed vigorously for 2 min and the aqueous phase was separated from the organic phase. The organic phase containing tocopherol radicals was dried over with $MgSO_4$ and measured using ESR spectroscopy along with aqueous phase containing ascorbyl radical.

4.3.5   Ascorbate and spin trapping studies on oxidative stress observed under ischemia/ anoxia and reoxygenation

Materials: Fetal bovine serum (FBS) was purchased from Biochrom (Berlin, Germany), medium and supplements came from PAA (Linz, Austria).

Cell culture

Human colon carcinoma cells (RKO) were cultured in Dulbecco's modified eagle medium (DMEM) with 4.5 g/l D-glucose. Medium was supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were transferred twice a week, and seeded 1×10^6 per 10 cm dish one day prior to the experiment. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. Hypoxia (0.5% O_2, 5% CO_2, 94.5% N_2) incubations were performed in a 3-gas incubator IG750 (Jouan, Unterhaching, Germany), and anoxia (0% O_2, 5% CO_2, 95% N_2) treatments were carried out in a flow-through manner in plexiglass chambers connected to a DIGAMIX 5KM 402 gas pump (Woesthoff GmbH, Bochum, Germany).

Experiment

Prior to the experiments medium was changed, and the cells were treated with 1 mM ascorbic acid (in PBS). The experiments were either conducted in normal DMEM medium (see above) or in serum-free Quantum 263 medium. Oxygen depletion treatment was conducted for 30, 60, 90 or 120 min. Alternatively, cells were reoxygenated after 60 min of anoxia treatment for additional 30 or 60 min. After the respective incubation the cells were harvested by washing them off the plates in medium and directly freezing them in liquid nitrogen. Measurements were performed using ESR spectroscopy

time [min]	0	30	60	90	120	60+10	60+30	60+60
	30	45	52	28	37			
normoxia	35	43	73	34	36			
	34	46	93	37	34			
ave.	33	45	73	33	36			
stdev.	2.6	1.5	20.5	4.6	1.5			
						reoxygenation		
hypoxia	30	52	50	37	38	73	157	67
	35	56	48	92	45	43	40	58
	34	49	48	65	49	57	101	60
ave.	33	52	49	65	44	58	99	62
stdev.	2.6	3.5	1.2	27.5	5.6	15.0	58.5	4.7
anoxia	30	42	43	40	43	62	72	60
	35	45	58	54	52	66	102	120
	34	53	52	48	58	68	136	61
ave.	33	47	51	47	51	65	103	80

Table 19 *Ascorbyl radical concentrations [nM] during normal, anoxia, hypoxia and reoxygenation conditions in RKO cell lines DMEM serum medium.*

ESR spectroscopy

Measurements were performed using E580 spectrometer from Bruker. The quantification of ascorbyl radicals was carried out by plotting the concentrations of a stable nitroxide radical (3-carboxyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl) dissolved in fresh 1% DMF as standard, against the area of ESR spectra after double integration. The resulting slopes were used to correlate ascorbyl radical concentrations, from its corresponding area after double integration. The area from ESR spectra was evaluated using commercial Microcal Origin software.

ESR : Scan time were varied in kinetic experiments and sweep width was changed to 100 G for DMPO and 8 G for ascorbyl radicals

<table>
<tr><td colspan="2" align="center">**E580**</td></tr>
<tr><td>Resonator</td><td>: dielectric</td></tr>
<tr><td>Microwave frequency</td><td>: 9.69 GHz</td></tr>
<tr><td>Microwave power</td><td>: 20.17 mW</td></tr>
<tr><td>Receiver gain</td><td>: 85</td></tr>
<tr><td>Mod. frequency</td><td>: 100 KHz</td></tr>
<tr><td>Mod. amplitude</td><td>: 1 G</td></tr>
<tr><td>No. of scans</td><td>: 5</td></tr>
<tr><td>Center field</td><td>: 3451 G</td></tr>
<tr><td>Sweep width</td><td>: 8 G</td></tr>
<tr><td>Resolution</td><td>: 512 points</td></tr>
<tr><td>Conversion time</td><td>: 163.84 ms</td></tr>
<tr><td>Time constant</td><td>: 81.9 ms</td></tr>
<tr><td>Sweep time</td><td>: 80 s</td></tr>
</table>

Table 20 *Instrumental setting used for ESR experiments.*

4.3.6   Fluorescent spin label (Dansyl-tempamine)

Materials: Dansyl chloride, tempamine from Sigma Aldrich Germany. Fluorescence spectrometer LS-5 luminescence Perlin Elmer.

Dansyl-tempamine was synthesized according to the modified procedure of *kalai, Hideg et al 2002*. 0.1M dansyl chloride and 0.1M TEMP-amine in chloroform was allowed to stay overnight. The raw product was dried over $MgSO_4$ and separated through an Al_2O_3 chromatographic column with $CHCl_3/CH_2Cl_2$ as moving phase.

To determine nitroxide reduction of fluorescent labelled spin probes in the presence of ROS, varying concentrations of ferrous ammonium sulphate was added to 50 µM of Dansyl-tempamine.

4.4   Fluorescent-labelling of ascorbic acid with N-methylisatoic anhydride

Materials: N-methylisatoic anhydride, Aldrich Germany. L(+)-ascorbic acid Riedel-de Haen, 4-(dimenthylamino) pyridine Fluka Germany.

4.4.1   Experimental protocol

0.1 M ascorbic acid and 0.1 M N-methylisatoic anhydride with catalytic amounts of 0.05 M DMAP in DMF containing 0.1 M triethylamine. The reaction was allowed to be heated up to 65 °C for 3 h in a reflux cooler until carbon dioxide evolution ceased. DMF was evaporated and the raw product mixed with sodium carbonate solution. The aqueous mixture was allowed to stand for 10 minutes and acidified with dilute phosphoric acid. The raw product was dissolved with ethyl acetate, dried over magnesium sulphate and evaporated under vacuum.

4.4.2   Thin layer chromatography, HPLC and MPLC

RP-18 TLC   : 5×7.5 cm, RP-18 $F_{254\,s}$ (Merck)

Moving phase   : acetonitrile / water 30: 70 %

fractions	R_f , λ = 254, 366 nm
raw	0.93, 0.67 and 0.52
fraction 2	0.93
fraction 3	0.67

Table 21 *TLC RP-18 of fractions of the raw product with acetonitrile/ water as moving phase.*

HPLC: Beckman, System Gold, 168 Detector, 125 Solvent Mode. Column; Lichrosorb RP-18 5 µm, 4×210mm, LKG Germany. Flow rate 1ml/min with acetonitrile/water as moving phase

MPLC: Pharmacia Biotech Pump P-50. **Column;** Lichrosorb® RP-1810 µm, 18×2 cm, Merck. Flow rate 3 ml/min with acetonitrile/water as moving phase

4.4.3   ^{1}H-NMR spectroscopy of fractions 2 and 3

For the structural determination of fractions 2 and 3 after chromatographic separations, ^{1}H-NMR spectrum was recorded with a 400 MHz Bruker spectrometer with tetramethylsilane (TMS) as an internal standard. Fractions 2 and 3 were dissolved in deuterium solvents like

D$_6$-DMSO or CDCl$_3$. The recorded ^{1}H -NMR spectra obtained were compared with the educts.

^{1}H-NMR	N-CH$_3$ [ppm]	4H aromatic ring [ppm]
N-methylisatoic anhydride	3.61	8.18 - 7.20

Table 22 *^{1}H-NMR of N-methylisatoic anhydride in CDCl$_3$.*

^{1}H-NMR [ppm]	2 C-OH	3 C-OH	4 C-H	5 C-H	5 C-OH	6 C-H$_2$	6 C-OH	N-CH$_3$	4H aromatic ring
fr. 2 DMSO-D$_6$	-	8.42	4.94	3.82	5.09	3.48	5.09	2.87	7.92-6.65
fr. 3 DMSO-D$_6$	11.13	8.43	4.8	4.14	5.45	4.27	-	2.85	8.43-6.56
fr.3 CDCl$_3$	-	-	4.78	4.49	-	4.30	-	2.89	7.90 -6.67

Table 23 *^{1}H-NMR of fraction 2 and 3 respectively. Chemical shifts in ppm.*

^{1}H-NMR [ppm]	2 C-OH	3 C-OH	4 C-H	5 C-H	5 C-OH	6 C-H2	6 C-OH
ascoh	11.01	8.3	4.72	3.73	4.87	3.44	4.87
fraction 2		8.42	4.94	3.8	5.09	3.5	5.09
Δδ H		0.12	0.23	0.04	0.22	0.06	0.22
fraction 3	11.13	8.43	4.8	4.14	5.45	4.27	
Δδ H	0.12	0.13	0.09	0.36	0.58	0.83	

Table 24 *^{1}H-NMR of fraction 2 and 3 respectively in relation to difference in the chemical shifts (ppm).*

4.4.4 Mass spectroscopy

Materials:
Mass spectrometer Finnigan MAT. EI-pos QT 200 70eV 1mA

no.	mass	intensity	% RA
1	75.9	3.77×10^4	2.5
2	77.0	3.42×10^5	22.4
3	78.0	2.22×10^5	14.5
4	79.0	6.70×10^4	4.38
5	91.0	7.70×10^4	5.03

6	91.9	6.02×10^4	3.94
7	103.9	5.61×10^5	36.71
8	105.0	6.11×10^5	40.02
9	106.0	1.92×10^5	12.55
10	107.0	4.91×10^4	3.22
11	131.9	3.38×10^5	22.12
12	132.9	2.10×10^5	13.75
13	133.9	8.08×10^5	52.92
14	134.9	6.24×10^4	4.08
15	150.9	6.57×10^5	42.99
16	151.9	6.66×10^4	4.36
17	264.8	8.52×10^4	5.8
18	308.8	1.99×10^5	13.02

Table 25 *Mass spectroscopy of the fraction 3.*

no.	Mass	intensity	% RA
1	73.0	6.29×10^6	5.45
2	76.9	1.81×10^7	15.68
3	78.0	8.34×10^6	7.24
4	79.0	5.04×10^6	4.37
5	91.0	4.88×10^6	4.24
6	103.9	2.58×10^7	22.42
7	105.0	2.84×10^7	24.61
8	106.0	1.24×10^7	10.74
9	115.9	2.89×10^6	2.51
10	115.9	5.12×10^6	4.44
11	131.9	2.56×10^7	22.22
12	132.9	1.61×10^7	13.94
13	133.9	1.15×10^8	100.00
14	134.9	9.54×10^6	8.28
15	149.9	4.41×10^6	3.83
16	150.9	6.05×10^7	52.45
17	308.8	7.38×10^7	67.94
18	309.8	1.19×10^7	10.36

Table 26 *Mass spectroscopy of the fraction 2.*

4.4.5 Hypoxanthine/ xanthine oxidase assay used for the oxidation of MANTA with the help of UV, fluorescence, and ESR spectroscopy

Materials: UV-spectrometer DU 640 Beckman. Fluorescence spectrometer LS-5 luminescence Perlin Elmer.

Protocol

1.07 mg/ml xanthine oxidase catalyst in 10 mM hypoxanthine substrate concentration was used as source of free radical generating system in 2.5 mM MANTA PBS medium dissolved in DMF or acetonitrile at pH 7.4 and measured using ESR spectroscopy as described in **section 4.3.5**

Hypoxanthine

- 14 mg hypoxanthine
- add to 10 ml PBS 7.4

EDTA-Na$_2$

- 37 mg EDTA-Na$_2$
- add to 10 ml PBS 7.4

(NH$_4$)$_2$SO$_4$ solution

- 26. 4 g (NH$_4$)$_2$SO$_4$ solution 2M
- add to 100 ml H$_2$O (deionised)

Enzyme sample

- 180 µl (NH$_4$)$_2$SO$_4$ solution 2M
- 20 µl XOD stock solution

Substrate buffer solution

- 9.8 ml PBS buffer
- 0.1 ml hypoxanthine suspension
- 0.1 ml EDTA-Na$_2$ solution 10 mM in PBS

For UV spectroscopic measurements, 50 µl enzyme sample was added to 2.95 ml buffer substrate solution and measured at $\lambda = 252$ nm for hypoxanthine every 2 second for 2 minutes or otherwise specified.

4   Summary and general discussions

Under physiological conditions oxygen is constantly being converted to reactive oxygen intermediates, in mitochondria, peroxisomes, cytochrome p450 systems, macrophages, neutrophils and in plasma membranes. These reactive oxygen species (ROS) are toxic and therefore alter cell integrity leading to cell damage. To protect itself against this toxic effect of ROS, living systems have developed defence systems that scavenge ROS formation. These systems include some enzymes, transporting proteins and small antioxidant molecules for instance vitamin C and E. This thesis describes a study on the antioxidant chemistry and activity of vitamin C *in vivo* and *in vitro* systems using ESR spectroscopy. Also, a new method was designed to label ascorbic acid with a fluorescent marker. Moreover, some important criteria were considered for the evaluation and quantification of ascorbyl radicals in human blood plasma using two types of ESR spectrometers.

The efficacy of heavy-dose vitamin C supplementation on healthy volunteers and patients undergoing coronary bypass surgery

The relevance of vitamin C in humans to protect the immune system, in collagen biosynthesis, and its antioxidant activity are well known. Although most of the animals can synthesize their own vitamin C, men among few other living creatures need it in their diet. Under stress conditions animals can produce a lot more vitamin C than normal values produced per day. This may show the requirement of vitamin C in the diet for living subjects that cannot produce it. A lot of controversies still exist on the amount of vitamin C required on a daily basis.

Although the dietary allowance of vitamin C intake recommended from US and European health authorities was about 100 to 200 mg per day, from a therapeutic point of view, the dietary recommendation of vitamin C was suggested to reach grams. Heavy doses of vitamin C are required mostly due to factors such as environmental influence, pregnancy and illness. Such heavy doses of vitamin C may also show pro-oxidant effects resulting from reactions with metal ions. Pro-oxidative effects of vitamin C stirred out a lot of discussions after a publication in Nature [Podmore et al 1998, Jenner 1998]. However other clinical trials showed inconsistent results showing antioxidant activity outweighing pro-oxidant effect.

The studies presented in the earlier stages of this thesis were to determine the efficacy of heavy-dose of vitamin C supplementation on healthy volunteers. The anti-oxidant defence mechanisms of free radical ascorbate oxidation were investigated using ESR spectroscopy and correlated with biochemical parameters for oxidative stress which were determined at the University of Hohenheim. In this study, healthy volunteers were intravenously given 750 mg or 7.5 g vitamin C. Ascorbyl radicals in plasma showed time-dependent changes before and after vitamin C infusion. These changes of ascorbyl radical concentration in plasma were compared with those of ascorbate during a time course of 8 hours. Apparently, ascorbate and ascorbyl radical concentrations showed similar time-dependent changes. Both these concentrations increased significantly at initial stages in relation to their respective doses that subsequently decreased steadily. Non-linear pathways showed that ascorbate oxidation did not end at the level of ascorbyl radical, but oxidized further to DHA within a short time interval. Therefore it is plausible that an increase in ascorbyl radical concentration as a result of ascorbate oxidation occurs during regular metabolic processes. The oxidation of ascorbate to ascorbyl radicals in healthy subjects may allow indirect estimation on the rate of oxidative transformations. Nevertheless, it should be noted that background ascorbyl radical ESR signals are usually seen at high ascorbate concentrations. This is consistent with the view that EDTA monovettes used for the removal of blood influenced plasma pH. Moreover, freezing and thawing of plasma resulted in an increase in ascorbyl radical concentrations caused by the presence of free metal ions.

Also, the time-dependent stability of ascorbyl radicals in plasma was investigated under laboratory conditions. Kinetic curves demonstrated that ascorbyl radicals decreased via first-order. Although, higher ascorbyl radical concentrations tend to show shorter half-lives. The changes in ascorbyl radical concentrations before and after vitamin C supplementation suggest ascorbate oxidations during normal metabolic processes in healthy subjects.

To prove the antioxidant and / or pro-oxidant activity of heavy doses vitamin C, secondary biochemical parameters for oxidative stress determined at the University of Hohenheim were compared with ascorbyl radical concentrations. However, the presence of higher levels of ascorbyl radicals in humans did not significantly affect Vitamin E, 8-oxoguanine and calcium levels in plasma. Indeed, malondialdehyde (MDA) levels decreased steadily after vitamin C therapy. These results suggest that, heavy doses vitamin C supplementation did not show any

pro-oxidant activity. Therefore, vitamin C can be considered to be a powerful antioxidant, implicating its importance in the protection against various oxidative stress-related complications.

Subsequently, the results acquired from healthy subjects after vitamin C supplementation enabled further research on oxidative stress with patients undergoing coronary aorta bypass operation (CABO). Previous publications have shown that bypass cardiac surgery can inflict damage to several tissues caused by the release ROS in response to inflammatory reactions. Moreover it is known that ROS generation takes place during myocardial ischemia and reperfusion in various experimental models and in human heart. ROS could therefore be responsible for bypass-induced damages or impairment of myocardial recovery. Therefore, the efficacy of vitamin C therapy was studied using ESR spectroscopy and compared with other parameters of oxidative stress.

Patients undergoing CABO were classified into placebo patients and those treated with 30g and 45 g vitamin C therapy in the form of bolus and continuous-infusion. Time-dependent ascorbyl radical levels in plasma as primary parameters of oxidative stress were correlated with ascorbate concentrations. Ascorbyl radical and ascorbate concentrations before and after vitamin C infusion showed rather similar time-dependent pathways. However, a marginal increase in ascorbyl radical concentrations in relation to ascorbate concentrations was seen during reperfusion and surgery. These results may reveal that ascorbate oxidation was induced by reactive oxygen species as a consequence of reperfusion or reoxygenation injury. Therefore, a correlation between ascorbyl radical concentration and ROS generation during periods of oxidative stress could be proved. To study pro-oxidative damage as a result of excess vitamin C therapy, secondary parameters for oxidative stress determined at the University of Hohenheim, such as MDA, intracellular adhesion molecules (sICAM), vascular adhesion molecules (sVCAM) and total antioxidant status (TAS) assays were correlated with ascorbyl radical concentrations. No adverse pro-oxidative effects on patients during reperfusion and bypass operation were observed. Indeed, MDA, sICAM and sVCAM levels were lower than placebo patients after vitamin C treatment.

Chemistry of ascorbate

In order to acquire an overview on the antioxidant activity of vitamin C, it is important to study the dynamic chemistry of free radical ascorbate oxidation using ESR spectroscopy. The different ionized forms of ascorbate have different redox properties and therefore the redox chemistry of ascorbate is highly pH dependent.

The data obtained by the studies on ascorbate oxidation in pH dependent buffers showed the dependence of ascorbyl radicals on the pH. Ascorbyl radical intensities increased steadily with increasing pH and increased significantly above physiological pH. Therefore, these results suggest that pH can greatly influence ascorbate oxidation caused by the step-wise ionization of the ene-diol group at the lactone ring. At pH values above 8, the dianionic form of ascorbate may outweigh the mono anionic form shifting its equilibrium to dehydroascorbate. Investigations on the dependence of pH on ascorbyl radical intensity are of great importance, since blood withdrawn in monovettes contains EDTA influenced plasma pH. Therefore, ascorbate oxidation must be carefully scrutinized by controlling the pH subsequently to eliminate experimental errors.

This part of the thesis discusses the ascorbate oxidation using chemical and enzymatic reactions as major source of oxygen free radicals. Generally, similar time-dependent changes in ascorbyl radical concentrations were observed during oxidation processes. However, the results show that the kinetics of redox reactions of ascorbate is also dependent on ascorbyl radical concentrations in the system. The data from ESR spectra showed that ascorbyl radical signal intensity as a function of pH, presence of trace catalytic metal ions, and ascorbate concentrations. This was in agreement with published papers in the past, e.g. Buttner et al 1993 and 1998. Under these circumstances, free radical ascorbate oxidation was easily studied when background levels are hardly visible in the ESR spectrum. This is achieved by removing traces of metal ions by passing ascorbate through resin columns and active coal. In experiments using hypoxanthine/ xanthine oxidase enzyme complexes as source of ROS, ascorbate oxidation showed more or less similar time-dependent patterns as seen in *in vivo* studies with healthy subjects. Also, the synergic effect of vitamin C and vitamin E in scavenging ROS was interesting enough to observe this mechanism in regular laboratory conditions. Time-dependent studies showed cross correlations, where the α-tocopherol radical concentrations formed after oxidation decreased in the presence of ascorbate in aqueous medium. This interesting phenomenon of one-electron redox transfer from α-tocopherol to ascorbate might be useful in a model system to show similar cascades of recycling processes taking place to eliminate radicals in biological systems.

Ascorbate oxidation during hypoxia, anoxia and reoxygenation

Since ascorbate is a major water-soluble antioxidant showing little or no toxic effects at high concentrations, it was an ideal choice as a natural non-invasive marker of free radical oxidations taking place in biological systems. *In vitro* studies showed time-dependent changes of ascorbate oxidation in RKO cell lines during hypoxia, anoxia and reoxygenation.

Interestingly, ascorbyl radical reactions during periods of oxidative stress revealed similar free radical ascorbate oxidation curves as seen in healthy subjects and patients after reperfusion and surgery. Generally, an increase in ascorbyl radical concentrations was observed after 30 minute periods of ischemia and reoxygenation. This reconfirms studies on oxidative stress that results in the release of free radicals during ischemic and reoxygenation injury.

Oxidative stress and spin trapping

An alternative method for determination of ROS *in vitro* was the application of spin trapping methods using ESR spectroscopy. DMPO was incubated in RKO cell medium following the same experimental procedure as with that of ascorbate. Paradoxically, all the collected spin trapped probes showed negative results. Time-dependent studies showed that FBS Serum in DMEM medium significantly enhanced paramagnetic nitroxide reduction. Further investigations carried out in serum-free medium turned out to be unsuccessful. In the last decade, extensive studies on oxidative stress using ESR spectroscopy were investigated with the help of spin trapping techniques to detect and identify free radicals in biological systems. However, numerous *in vitro* studies on spin trapping previously and in this work showed its limitations in the determination of free radicals.

Fluorescent-labelled ascorbic acid and its redox chemistry

An alternative strategy to determine ROS is the synthesis of new fluorescent-labelled ascorbic acid. This modified compound may open new pathways in determining free radical species using ESR as well as fluorescence spectroscopy. In addition, fluorescence spectroscopy may overcome the detection limits of ESR spectroscopy. Moreover, due to reduced or no toxicity of ascorbic acid, real-time measurements in the detection, interaction and co-localization of ROS using fluorescence confocal laser microscope can be achieved in the near future.

A new fluorescent-labelled ascorbic acid ester (MANTA) was successfully synthesized from N-methyl isatoic anhydride and ascorbic acid. The multifaceted antioxidant character of

MANTA makes it challenging for studies using UV, ESR and fluorescence spectroscopy. MANTA is relatively stable in organic solvents like acetonitrile and DMF, unstable in DMSO and partly soluble in water. This fluorescent-labelled ascorbic acid ester was isolated by HPLC, purified and characterized using ¹H-NMR and mass spectroscopy. Time-dependent fluorescence intensity and radical concentrations after oxidation revealed unique structural and dynamic information. MANTA showed that it's modified structure, and redox properties towards oxidants do not differ from those of native ascorbate, particularly, since both compounds demonstrated similar properties such as pH dependency, type of ESR structural information of radical species and time-dependent oxidation kinetics. ROS can be easily detected by MANTA using chemical and enzymatic reactions. Another unique feature of MANTA is the emission of an intense blue colour, which can be useful in future for real-time detection in cells using fluorescence confocal laser microscopic techniques. Moreover ROS can be easily targeted by observing areas of fluorescence quenching. Therefore by the application of ESR and fluorescence spectroscopic methods for targeting and determining ROS, MANTA may enhance and open new strategic pathways for studies on oxidative stress responses in biological systems. Nevertheless, studies on MANTA showed that this molecule was labile in aqueous medium. MANTA was relatively stable in reaction medium containing more than 5% acetonitrile or DMF and very unstable in DMSO. Therefore, studies on the antioxidant activity of MANTA in organic solvents may turn out to be inadequate in biological systems in response to cell viability.

Quantification and evaluation of ascorbyl radicals using ESR spectrometers

In the course of this thesis a Bruker high-end research ESR spectrometer (E 500 Series), employed for the determination and quantification of ascorbyl free radical concentrations in plasma was compared with two low cost bench top ESR spectrometers. The radical concentrations determined with the *e-scan* spectrometer were consistently higher as compared to the values determined with the E580. The rapid sampling method of *e-scan* in relation to the E580 spectrometer helped in gaining measuring time and thus compensated the low stability of ascorbyl radicals.

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8 Lebenslauf

Name	:	Fabian Rozario
Geburtsdatum	:	19.11.1961
Geburtsort	:	Trichur (Indien)
Anschrift	:	Berliner Str 55
		66849 Landstuhl
Staatsangehörigkeit	:	indisch
Schulbildung	:	1980, Model High School for Boys, Trichur
Studium	:	1982, Pre-Degree, University of Calicut, India
		1985, Bachelor of Science, University of Kerala, India
	:	1989, Nachweis deutscher Sprachkenntnisse (P.N.D.S), Kaiserslautern
		1994, Diplomvorprüfung Chemie, TU Kaiserslautern
		2000, Diplom Chemie, TU Kaiserslautern
		2004, Promotionstudium, Arbeitskreis Prof. Dr. W.E. Trommer Fachbereich Chemie, TU Kaiserslautern