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DIPLOMARBEIT

Titel der Diplomarbeit

„Studien an Cytochrome c: Elektronen Abgabe, Zerfall, Wechselwirkungen mit Ascorbin Säure und Experimente in vitro“

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Cytochrome c studies: Electron Emission, Degradation, Mutual Interaction with Ascorbic Acid and Experiments in vitro.*

by Günter Walder

* The investigations were performed in the frame of the research project: "Free Radicals action on Sexual Hormones in Respect to Cancer", under the supervision of Emer.o.Univ.Prof.Dr. Nikola Getoff and financial support by the Austrian Science Fund (FWF).

Contents

List of Abbreviations

$3xdH_2O$	triply distilled water
AA	amino acids
Ar	argon
ATCC	American Type Culture Collection
BSA	bovine serum albumin
ClEtOH	chloroethanol
CO_2	carbon dioxide
CYC	cytochrome c
CYP	cytochrome P450
D-Glc	D-glucose
DMEM	dulbecco's modified eagle medium
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
e^-	electrons
e^-_{aq}	solvated/hydrated electrons
EtOH	ethanol
FCS	fetal calf serum
Fe	iron
$HClO_4$	perchloric acid
HPLC	high performance liquid chromatography
I_0	Intensity of the monochromatic UV-source
L-Gln	L-glutamine
MCAA	monocholoracetic acid
MW	molecular weight
NaCl	sodium chloride
N_2	nitrogen
PBS	phosphate buffered saline
VitC	ascorbic acid (vitamin C)

Chapter 1

Introduction

It has been previously experimentally shown that organic compounds in aqueous solutions having substituents, such as:

- $-OH$
- $-OCH_3$
- $-NH_2$
- $-NHCH_3$
- $-COOH$
- $-OPO_3H_2$ etc.

can eject "solvated" electrons (e^-_{aq}) when excited into the singlet state [1]. The yield of the e^-_{aq} depends on the substrate concentration, pH, temperature and absorbed energy. This effect has been recently observed also on hormones [2], vitamins [3] and other substances of biological interest.

In the organism e^-_{aq} ejection and transfers are very important processes. They are also determining factors in various metabolic systems, whereby the needed energy is mainly provided by enzymes. For studies in the laboratory monochromatic UV-light (λ = 254 nm; E = 4.85 eV $h\nu^{-1}$; 1 eV = 23 kcal mol^{-1}) was used [2, 3].

The e^-_{aq} represents a thermalized electron (E $\leq$ 10 eV) surrounded by the positive dipoles of water molecules (or other polar liquids) within less then 2 ps. e^-_{aq} is

a strongly reducing entity, representing the basic form of the H-atom:

$$OH^- + H \longrightarrow e^-_{aq},\ k = 2.2 \times 10^7\ \ L\ mol^{-1}\ s^{-1}[4] \tag{1.1}$$

$$H^+ + e^-_{aq}\ \longrightarrow H,\ \ k = 2.3 \times 10^{10}\ L\ mol^{-1}\ s^{-1}[4] \tag{1.2}$$

The e^-_{aq} has been discovered by the conversion of CO_2 into simple organic substances, such as aldehydes and carboxylic acids under the influence of γ-ray and UV-light [5, 6], e.g.:

$$CO_2 + e^-_{aq} \longrightarrow {}^{\bullet}COO^-, \qquad k = 1 \times 10^{10} L\ mol^{-1}[4] \tag{1.3}$$

$$CO_2 + H\ \longrightarrow {}^{\bullet}COOH/HCOO^{\bullet}, \quad k = 8 \times 10^6\ L\ mol^{-1}[4] \tag{1.4}$$

$$2\ {}^{\bullet}COO^-\ \longrightarrow (COO^-)_2\ (oxalate), \ k = 2 \times 10^9\ L\ mol^{-1}[4] \tag{1.5}$$

$$2\ {}^{\bullet}COOH \longrightarrow (COOH)_2 \qquad (oxalic\ acid) \tag{1.6}$$

$$\longrightarrow CO_2 + HCOOH \qquad (formic\ acid) \tag{1.7}$$

The absorption spectrum of e^-_{aq} has a maximum at $\lambda_{max} = 720$ nm, and was measured by pulse radiolysis [7]. Using electron-spin-resonance method (ESR) a $\lambda_{max} = 575$ nm of trapped electrons (e_{th}) in aquesous alkanline ice-matrix (77° K) was observed [8].

1.1 Cytochromes

As mentioned above, enzymes act as the main energy source for the generation of e^-_{aq} in the organism. In order to learn more about the thereby involved reaction mechanisms, cytochrome c (CYC) was chosen as a model for the studies.

Cytochromes are a large group of proteins with vital functions within organisms. The name cytochrome derives from the greek "kytos", which means body and in biological meaning cell, and "chroma" what is color. This implicates that cytochromes are colored proteins. Cytochromes contain heme as prosthetic group and are classified according to the heme and their spectrophotografic behaviour. Hemes are tetrapyrollic molecules with a lot of different biochemical functions (for basic structure see Figure 1.2a). Variations of hemes with their binding partners

are mentioned in Table 1.1. Within cytochromes most of the hemes have a Fe^{2+} center, which can either serve as e^--donor or acceptor, by being oxidized to Fe^{3+} (Equations 1.8 and 1.9). This property makes cytochromes very potent substances within e^--transport chains, like for example cytochrome c (CYC) in the respiratory chain [9, 10] or the cytochrome-b_6/f-complex at the e^--transport during photosynthesis [11], where they are steadily reduced and reoxidized, maintaining the e^--flux. Another important function of heme is the O_2 transport in the blood, where heme b is the prosthetic group of hemoglobin, binding O_2 or CO_2 within erythrocytes [12]. Heme b is the prosthetic group of the cytochromes of the P450 family (CYP) too, which are usually not part of electron transport chains but oxygenases and are acting as potent e^-_{aq} -donors. CYP are of interest for these studies, because of their role in metabolizing water unsoluble substances and interactions with hormones and drugs.

$$Fe^{2+}_{aq} \rightsquigarrow h\nu(Fe^{2+}_{aq})^* \longrightarrow Fe^{3+}_{aq} + e^-_{aq}\ (e^- donor) \tag{1.8}$$

$$Fe^{3+}_{aq} + e^-_{aq} \longrightarrow Fe^{2+}_{aq}\ (e^- acceptor) \tag{1.9}$$

Table 1.1 – Samples of heme with some of their respective partners.

heme	compound of
heme a	cytochrome c oxidase
heme b	hemoglobin, myoglobin, cytochrome c reductase, cytochrome P450
heme c	cytochrome c, cytochrome cd_1
heme s	chlorocruorin
heme P460	hydroxylamin oxidoreductase [13]
siroheme	ferredoxin nitrite reductase, sulfite reductases of plants

1.1.1 Cytochrome P450

CYP are proteins with a sequence of about 500 amino acids (AA) and heme b as prosthetic group (Figure 1.2b). CYP is a large family of intercellular heme proteins, with about 1000 members including organisms from bacteria, plants and animals. There are more than 50 different CYP known in humans so far, playing a crucial role in:

(a) drug metabolism
(b) metabolism of pollutants and xenobiotics
(c) biosynthesis of hormones and
(d) metabolism of fat soluble vitamins.

Regarding the objectives in the frame of these studies, point (b) "metabolism of xenobiotics" [14] and point (c) "biosynthesis of hormones" are making CYP an interesting compound of the experimental set up. The pathway of hydroxylation of progesteron [15] or synthesis of dopamin by CYP [16] are so far well understood and as those hormones are part of experiments within the project, it is obvious to have a close look at CYP as well. Progesteron and other hormones, like estradiol, dopamin, testosteron or adrenalin were already shown to be able to emit e^-_{aq} [17, 18, 19, 20] and to be regenerated by strong antioxidants, like ascorbic acid (VitC), as potent e^-_{aq} donor [21].

When hormones are released within an organism due to environmental properties, they need to interact either with their designated substances or to bind to their corresponding receptors, to start the proper mechanisms as response. The potential of hormone receptors to emit e^-_{aq} and the possibility of interactions via e^-_{aq} has been shown recently [22]. A possible mechanism of hormones and its emitted e^-_{aq} has been suggested by N. Getoff [2], where e^-_{aq} may have a priming function, preparing the hormones binding partners for the arrival of the hormones, leading to a way faster response of downstream reaction cascades (Figure 1.1). The way of interaction of CYP with hormones is well studied. The communication process via the brain, based on the specific frequenzy of the emitted e^-_{aq} is also reported [23], where e^-_{aq} communications are a necessary part of this interactions.

Within cytochromes, the prosthetic group, the heme, is necessary for e^--transfers. The Fe^{2+} serves as e^- donor or Fe^{3+} as an acceptor and both are of great importance by the determination of the e^-_{aq} -quantum yield. As already mentioned, CYP have heme b as prosthetic group. Within all groups of heme, heme b is the most common in biological systems. It is also known as iron-protoporphyrin IX or protoheme IX. The binding to the protein could either occur by a thiol group of a cystein or via nitrogen from the imidazol group of a histidine, which is more common in mammals. They directly serve as ligand to the heme-iron and there is

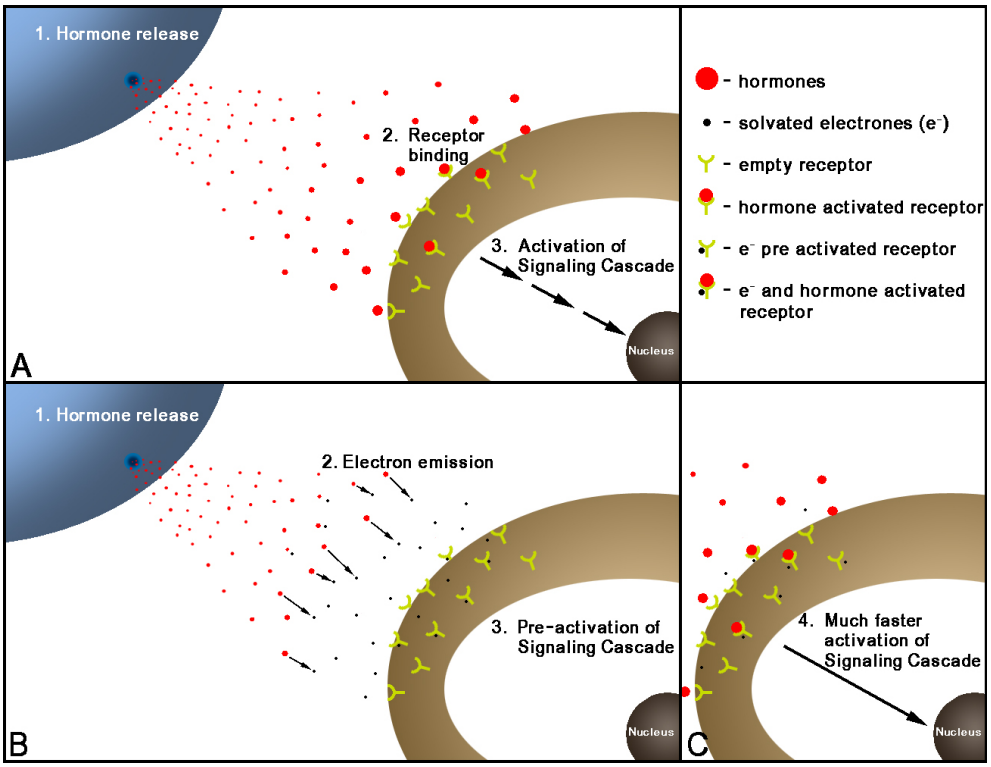


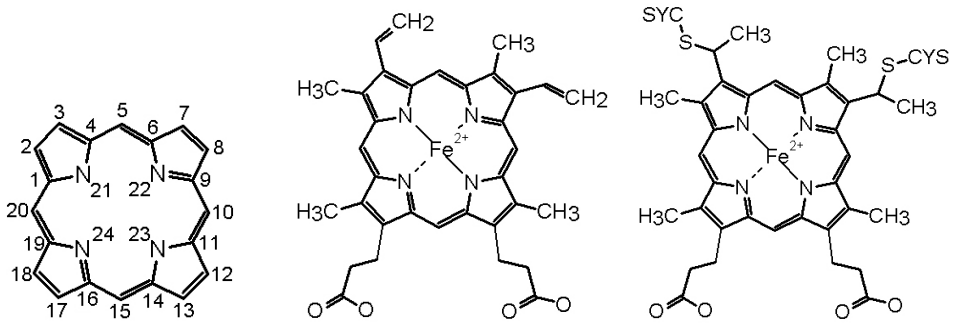
Figure 1.1 – Model of e^- priming of receptors during hormone release. (A) Usual mode of activation of signaling cascades after hormone release and receptor binding. (B) Hormones are releasing e^-, which are arriving at their receptors way faster and therefore preparing the receptors for the arrival of the hormones. (C) When hormones arrive and bind to their designated receptors signaling may occur much quicker, due to the priming of receptors after e^- binding.

no covalent bond between the heme b and the protein matrix.

For experiments in situ as well as in vitro a large amount of CYP would be needed. Therefore, CYC was chosen as model for present CYP investigations.

1.1.2 Cytochrome c

CYC is a small protein consisting of 104 AA and a heme c as prosthetic group (see Figure 1.2c). As it is expected that the heme ring of CYC will contribute to most of the emitted e^-_{aq}, the differences between heme b and the model heme c should be recognized as well (see Figure 1.2). In contrast to heme b in CYP, the heme c is bound by two covalent bonds to the protein matrix via the thiol groups of two



(a) Porphyrin ring **(b)** Heme b **(c)** Heme c

Figure 1.2 – Porphyrin ring and Heme b vs Heme c.

cysteins at C 3 and C 18 of the porphyrin ring. The iron core is easily reduced to Fe_2^+ (e_{aq}^- -donor) or oxidized to Fe_3^+ (e_{aq}^- -acceptor) what makes CYC a potent e^- donor or acceptor. In the presence of air peroxyl free radicals are formed:

$$O_2 + e_{aq}^- \rightarrow O_2^{\bullet -}, \qquad (k = 1.9 \times 10^{10} mol.L^{-1}.s^{-1}) \tag{1.10}$$

$$O_2 + H \ \rightarrow HO_2^{\bullet}, \qquad (k = 2 \times 10^{10} mol.L^{-1}.s^{-1}) \tag{1.11}$$

$$HO_2^{\bullet} \quad \rightleftharpoons H^+ + O_2^{\bullet -}, \ pK = 4.8[24] \tag{1.12}$$

The mechanisms of CYC within the mitochondrial electron transport of the cellular respiration are well understood (see Figure 1.3) and it also has a major function in formation as well as scavenging of reactive oxygen species [25, 26]. Another important part is the participation of CYC in apoptosis, the programmed cell death. CYC is usually located at the intermembrane space site of the inner mitochondrial matrix within the respiratory chain complexes, but when released from mitochondria to the cytoplasm, it binds to apoptotic protease-activating factor 1, leading to formation of the apoptosome and cell death [27, 28, 29, 30].

The role of CYC within the electron transport chain is the e^- transfer from complex III to complex IV. The initial step leads to two CYC_{ox} (Fe^{3+}) getting oxidized by one ubichinol, which leads to two CYC_{red} (Fe^{2+}) and ubichinon in complex III. This is done by the Ubichinol:Cytochrome c-Oxidoreductase, also known as "Cytochrome c Reductase" (equation 1.13). The CYC_{ox} theirselves are now going to complex IV where they are reduced by the Cytochrome c Oxidase to CYC_{red}, and one e^- per CYC is transferred to the substances participating in

complex IV, like cytochromes a and a_3 or copper-ions (equation 1.14). At the end of the e^--transport-chain one water molecule is released within the mitchondrial matrix while ten protones are pumped to the intermembrane space to maintain the electrochemical gradient used by ATPases for phosphorylation of ADP to ATP. The bonding of one phosphate to one ADP uses about 3 protones streaming back to the inner matrix through the complex of the ATPase, pushing the ATP production [31]. ATP is one of the most important energy sources of the cell, supporting lots of metabolic reactions by providing energy due to dephosphorylation to ADP or AMP [32].

$$Ubichinol + 2\ CYC_{ox} \longrightarrow 2\ Ubichinon + 2\ CYC_{red} \tag{1.13}$$

$$2\ CYC_{red} + 1/2\ O_2 + 2\ H^+ \longrightarrow 2\ CYC_{ox} + H_2O \tag{1.14}$$

Aside being reduced within different enzyme complexes, it is easily possible to produce CYC_{red} by using antioxidants like ascorbic acid (VitC) in situ. This effect was utilised during the experiments, for steady regeneration of CYC during irradiation influences.

1.2 Antioxidants

Antioxidants are very important substances in nature. Naturally occurring antioxidants are for example VitC, tocopherols, flavones, or phenolic contents of plants. They are very reactive against electrophils and inhibit oxidative processes by scavenging of oxidizing radicals formed during metabolism processes or due to environmental influences, often acting via H-transfers. Basic reactions are given in equations 1.15 and 1.16

$$R^\bullet + XH_2 \longrightarrow XH^\bullet + RH \tag{1.15}$$

$$ROO^\bullet + XH_2 \longrightarrow XH^\bullet + ROOH \tag{1.16}$$

where XH_2 is the antioxidant (e.g. VitC).

The developed $XH^\bullet$ is chemically either very inert (phenolic antioxidants) or

neutralized by radical combination (1.17) or disproportionation (1.18).

$$R^{\bullet} + XH^{\bullet} \longrightarrow R - XH \tag{1.17}$$

$$XCH_2CH_2^{\bullet} + XCH_2CH_2^{\bullet} \longrightarrow XCH_2CH_3 + XCH_2 = CH_2 \tag{1.18}$$

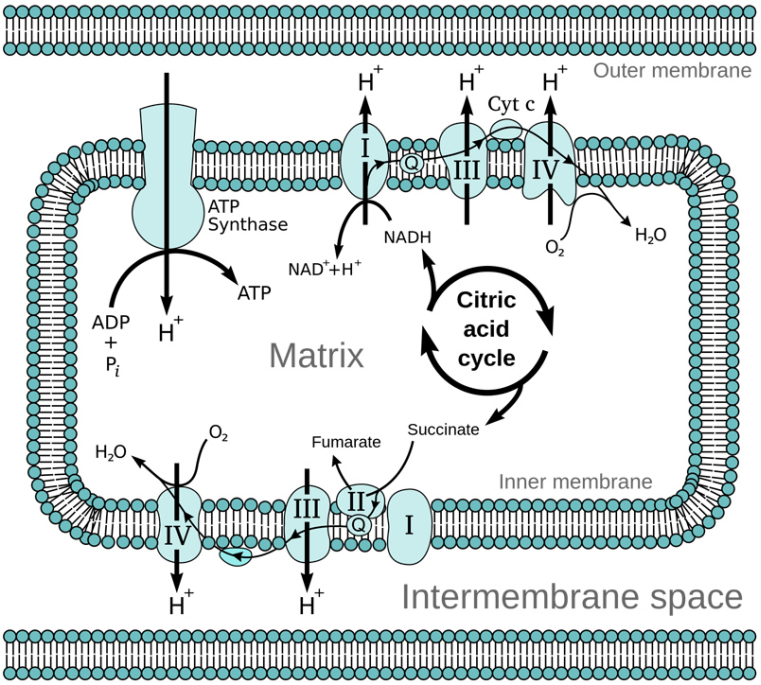


Figure 1.3 – Mitochondrial electron transport chain. This figure shows the respiratory chain within the inner membran of mitochondria building up the electrochemical potential needed for ATP synthesis through ATPases. Starting with products from the citric acid cycle, succinat and reduced Nicotinamide adenin dinucleotide (NADH), there are two possible ways for maintaining the electrochemical gradient. One is using succinat as e^- source (lower chain), the other using NADH (upper chain). e^- are gained by oxidation of succinat and NADH to fumarat and $NAD^+ + H^+$. This causes protones to be pumped into the intermembrane space of the mitochondria and the resulting e^- to enter the electron transport chain. The migration of the e^- throughout the chain is achieved by constant oxidation and reduction cycles of the participating complexes, with steady protone pumping into the intermembrane space. Cytochrome c is located at the intermembrane space site of the inner membrane, sustaining the e^- flux between complex III and IV. At the end of the chain O_2 is hydrogenated. The H^+ gradient is now used by ATPases for phosphorylation of ADP and P_i to ATP, using three H^+ per ATP. Source: "Mitochondrial electron transport chain-Etc4.svg", http://en.wikipedia.org/wiki/File:Mitochondrial_-electron_transport_chain%E2%80%94Etc4.svg, 2013-09-09.

1.2.1 Ascorbic Acid

In addition to tocopherols, ascorbic acid, also known as vitamin C, is one of the most important antioxidants. It plays also a vital role within several hydroxylation reactions, where it acts as e^- donor and has a crucial function at hydroxylation of prolyl within collagen, keeping the needed Fe in the Fe^{2+} state [33]. VitC is acting within a redox system being reduced to dehydroascorbic acid from VitC via two 1-e^- -transfers. Its major functions as antioxidant are:

(a) reduction of superoxid radicals (e.g. in chloroplasts),
(b) protection of membrane lipids and
(c) scavenging radicals.

VitC is reducing carbon-centered radicals via H-transfer, see equation (1.15). The biologically active form, the e^-_{aq} donating form, of VitC is the ascorbat, which is able to emit two e^-_{aq} within a two step reaction leading to dehydroascorbic acid via an intermediate ascorbyl radical (Figure 1.4) [34, 35]. Dehydroascorbic acid is usually regenerated to VitC within a redox system.

The high regenerative and antioxidative potential has been shown in several studies [3, 37] and its radiation-protective effect is well studied and described [38]. Interactions of VitC with chemotherapeutics like Mitomycin C [39, 40] and the impact of VitC on hormone treatment under irradiation influences were also explored recently [41, 42]. It was shown that VitC is able to regenerate Mitomycin C and hormones through electron transfer, when being in mixture with one of the substances. These are important discoveries concerning the fact that this substances are often included in cancer therapies. Mitomycin is applied as chemotherapeutic and hormones (especially sex hormones) are often used for supplementary therapy in combination with radiotherapy. Taking all this facts together, new strategies of treatment could be found.

1.3 Study Objectives

This diploma thesis was part of the project "Free Radical Action on Sexual Hormones in Respect to Cancer", Contract No. P21138-B11 (head of the project:

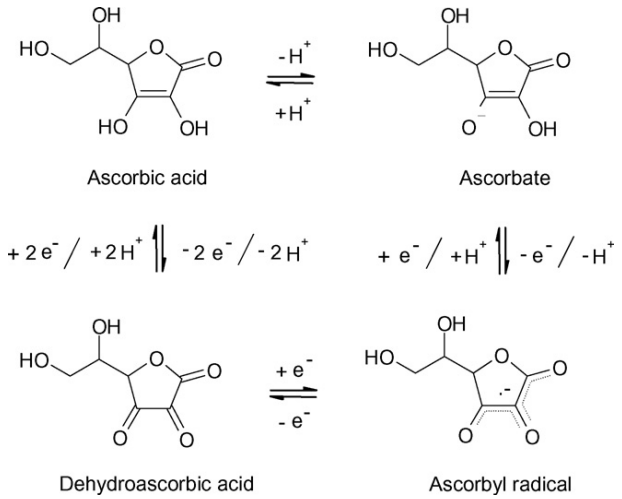


Figure 1.4 – Ascorbic acid and its biologically active forms. Ascorbat is the central state being able to emit two e^-_{aq} via formation of the intermediate Ascorbyl radical and resulting in dehydroascorbic acid. The emitted e^-_{aq} are leading to most of the important functions of VitC in biological systems. In biological sytems dehydroascorbic acid is regenerated to VitC. Source: Dr. Heike Schittl; "Electron Emission and Biological Consequences of Hormones in Polar Media, Studied on Testosterone, Progesterone, 17β-Estradiol and Genistein. 2011" [36]

Prof. Dr. N. Getoff), funded by the Austrian Science Fund (FWF).

In addition to determination of the quantum yield of e^-_{aq} ($Q(e^-_{aq})$, experiments in vitro were performed using MCF-7 breast cancer adenoma cells as a model in order to get a deeper understanding of the subject matter.

The objectives of the present work concern also investigations for the explanation of the multifaceted mechanisms of cytochrome, as well as some cytochromes of the P450 family, which are known to interact with hormones. Therefore, CYC was used as model under the influences of radiation and interactions with VitC. For simulation of the biological process monochromatic UV-light (λ = 254 nm; E = 4.85 eV $h\nu^{-1}$; 1 eV = 23 kcal mol^{-1}) was used for excitation of the substrate in single state. In addition also γ-irradition was used in the frame of experiments in vitro. The e^-_{aq} emission is associated with the formation of free radicals. Their behaviour in mixed solutions, containing VitC, under irradiation was also purpose of the studies. Experiments were performed in situ by using HPLC method for determination of the final products as well as in vitro using MCF-7 breast cancer

cells as an appropriate model in order to learn about the biological effects.

The objectives of this diploma thesis can be summarized in the following points:

(i) studies to prove the ability of CYC as e^-_{aq} donor and the conditions for verify this ability,

(ii) studies of mutual interaction of CYC and VitC and transfer of e^-_{aq} between the substances,

(iii) in vitro studies with MCF-7 cells and γ-irradiation to prove the ability of CYC of being an irradiation protectant and

(iv) in vitro studies with MCF-7 cells and γ-irradiation to prove mutual interactions of CYC and VitC and their impact on cell survival 1.5.

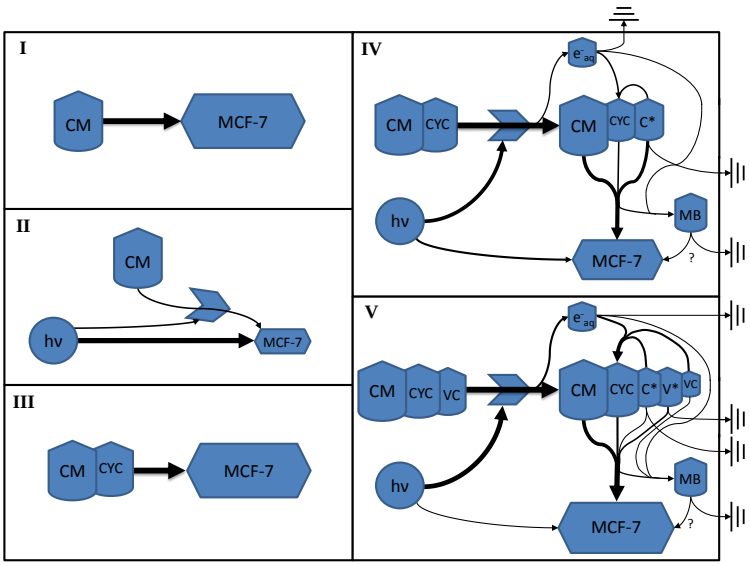


Figure 1.5 – Odum diagram of possible reaction ways at in vitro experiments. (I) MCF-7 cell culture with cell culture medium (CM) only. (II) Under irradiation conditions ($h\nu$) MCF-7 cells will die. (III) MCF-7 in culture with CYC behave same way like growing in culture media only. (IV) When irradiated with γ-rays in medium containing CYC, CYC acts as a protection reagent, attenuating the irradiation impact by defusing resulting radicals. Degradation products of CYC or VitC (C* and V*) might form other metabolites with no high impact on cell culture growth. (V) After adding VitC (VC), resulting C* are quickly regenerated by the high reduction power of VitC. This way higher yields of the applied CYC can be used to reduce the irradiation impact, resulting in higher survival rates.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals used were of high grade purity and for analytical purposes. Triply distilled water (3xdH_2O) was provided by a Milipore Direct-Q UV3 and applied for the preparation of solutions. For origins of the applied chemicals and materials see tables 2.1 and 2.8.

Table 2.1 – Materials. Productnumbers and origins.

Materials	Prod.No.	Company
1 x Trypsin EDTA, 0.05 %	25300	GIBCO
2-Chloroethanol, 99 %	18.574-4	Sigma Aldrich
Acetonitril	8825.2	ROTH
Argon	51380812	Messer
BSA	N208-10G	VWR
CO_2	51838266	Messer
Cytochrome c, from bovine heart	C2037	Sigma Aldrich
D-glucose	1.08337	MERCK
DMEM 1x	11880	GIBCO
EtOH 96 %	D56.10	LACTAN
EtOH absolute p.a.	1.00983	MERCK
FBS, qualified	26140079	GIBCO / Invitrogen
Formic acid 98 - 100 %	K02918284	MERCK

Table 2.1 – Materials. Productnumbers and origins.

Materials	Prod.No.	Company
Hg(II)-thiocyanate	4484	MERCK
Insulin, from bovine pancreas	I5500	Sigma Aldrich
Iron-(III)-nitrate nonahydrate	CN84.1	ROTH
L-ascorbic acid	A5960	Sigma Aldrich
L-glutamine	G3126	Sigma Aldrich
NaOH	10644981000	MERCK
PBS, pH 7.4, 10x	70011	GIBCO
Perchloric acid 60 % p.a.	1.00518.1001	MERCK
NaCl	3957.1	ROTH

2.2 Sterilisation

Materials as well as solutions used for in vitro experiments, like DMEM, FCS or PBS, were sterile one way products. Chemicals used for cell culture experiments were first dissolved in a minimum amount of dilution liquid and sterile filtrated, before being added at the right amount to a solution or culture.

Glassware was cleansed by a Miele dishwasher, Mielabor G 7783, and autoclaved with a Varioclav for 20 minutes at 121° C and 2 bar pressure.

2.3 Radiation

Experiments were done with two different irradiation sources:

- monochromatic UV-light (λ = 254 nm; E = 4.85 eV $(h\nu)^{-1}$)
- ^{60}Co -γ-ray

2.3.1 UV Irradiation

Monochromatic UV-light (λ = 254 nm) was used in the frame of experiments for:

(i) excitation of CYC in single state resulting in emission of e^-_{aq} ,

(ii) determining of the thereby formed final products by HPLC.

2.3.1.1 UV Irradiation Source

As UV source an "Osram" HNS 10 W, Hg-low-pressure lamp, with incorporated Vycor filter for elimination of the 184.9 nm line, was used to get monochromatic UV light with a wavelength of λ = 253.7 nm (E = 4.85 eV/hν; 1 eV = 23 kcal mol^{-1}).

2.3.1.2 Irradiation Vessel

For irradiation experiments using UV light a special irradiation vessel with 4π-geometry was used, see Figure 2.1. The 4π-geometry is providing uniform irradiation yields of the solution under stirring [43, 44, 45].

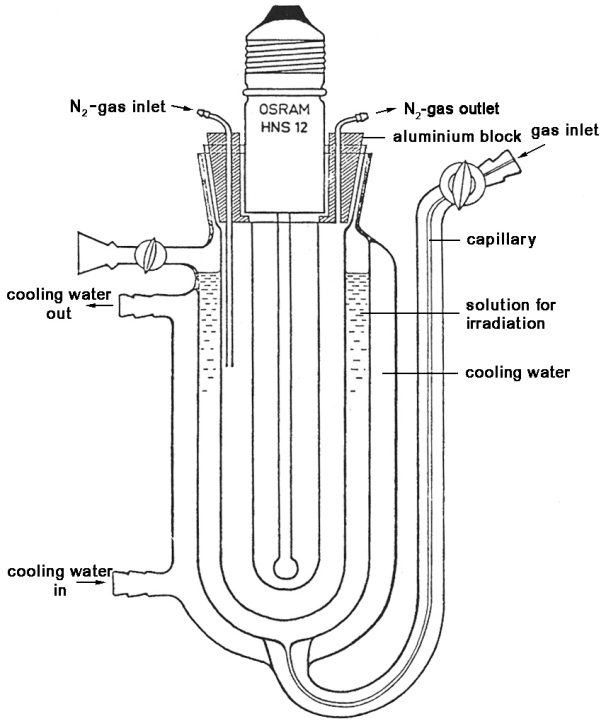


Figure 2.1 – Irradiaton vessel with 4π-geometry. Uniform irradiation yields are ensured by the centered UV-light-source. The special double wall design enables working at constant temperatures through a thermostat. The possibility for saturating the probes with different gases (eg. Ar, O_2, N_2, NO_2) is provided by the gas in- and outlet.

2.3.1.3 Actinometry

Actinometric measurements were performed in order to determine the intensity (I_0) of the monochromatic UV-source (λ = 254 nm) in connection with the 4π-irradiation vessel. As specific actinometer an aqueous solution of 2.5 mol L^{-1} monochloracetic acid (MCAA) was applied (MCAA actinometry) [46, 47]. For this purpose two calibration curves were needed:

1. Curve A: (OD cm^{-1}) at 460 nm versus Cl^- (mol L^{-1}), (Figure 3.1) and
2. Curve B: Cl^- (mol L^{-1}) versus irradiation time (min), (Figure 3.2).

The spectroscopic measurements were undertaken at 37° C (organism temperature). I_0 at λ = 253.7 nm was determined based on the reaction:

$$e^-_{aq} + ClCH_2CH_2OH \longrightarrow Cl^- + {}^{\bullet}CH_2CH_2OH \tag{2.1}$$

$$Q(Cl^-) = Q(e^-_{aq}) \tag{2.2}$$

The same UV light source as well as the same UV irradiation vessel were used throughout all experiments.

2.3.1.4 Calibration

The absorbed UV-light at 253.7 nm in the 4π-geometry (see 2.1) is partly photolysing the actinometer under Cl^- formation. The yield of the resulting Cl^- is proportional to the UV-quanta absorbed, therefore making it possible to determine the corresponding UV-intentsity (I_0) at 37° C. Samples of irradiated solution were taken and mixed with a solution containing iron-(III)-nitrate and Hg(II)-thiocyanate according to the method by Florence and Fara (1971) [48]. The solutions together with the developed Cl^- are leading to the formation of coloured iron-thiocyanat complexes (Equations (2.3)). This process is spectrophotometrically detectable and the Cl^- yield definable with the help of a NaCl calibration line. With those parameters it is possible to calculate the intensity I_0 of the UV-light lamp.

$$\begin{aligned} 2Cl^- + Hg(SCN)_2 &\rightleftharpoons HgCl_2 + 2SCN^- \\ 4Cl^- + Hg(SCN)_2 &\rightleftharpoons HgCl_2^{-4} + 2SCN^- \\ SCN^- + Fe^{2+} &\rightleftharpoons Fe(SCN)^{2+} \end{aligned} \tag{2.3}$$

2.3.1.4.1 NaCl Calibration Curve - Procedure (Curve A)

The calibration curve is showing Cl^- mol L^{-1} as a function of UV quanta absorbed, due to the fact that the Cl^- yield is proportional to the absorbed UV quanta as noted above.

Reagents used for the NaCl calibration line:

- 1 L 1 x 10^{-4} mol L^{-1} NaCl in 3xdH_2O as standard solution
- Solution A (50 ml)
- Solution B (50 ml)

Solution A:

			50 ml:		
0.375	M	Fe(III)-Nitrate	7.575	g	Fe(III)-Nitrate
5.250	M	$HClO_4$	28.530	ml	60 % $HClO_4$ in 3xdH_2O

Dissolve Fe(III)-Nitrate in 60 % $HClO_4$ in the exhaust hood and fill the remaining volume up to 50 ml with 3xdH_2O. Store for a maximum of 3 days at 4° C.

Solution B:

Dissolve Hg(II)-thiocyanate in 98 % EtOH p.a. until solution is fully saturated. Store at 4° C.

Establishing the NaCl calibration line:

- Take 1, 3, 5, 7 and 8 ml from the standard solution and transfer it to a 10 ml flask.
- Add one ml of solution B and afterwards one ml of solution A to each of the flasks and fill the flasks with 3xdH_2O up to 10 ml. Make a blank with water and solution B and A only as well. Mix the solutions well. The order of adding solution B and A is important for better reproducibility and should be done in this order throughout all experiments.
- Determine the extinction at λ = 460 nm after 5 minutes, use 3xdH_2O as reference.
- Calculate ΔOD_{460} by substracting OD_{460} of the samples from the OD_{460} of

the blank.

- Create a calibration line OD_{460} nm cm^{-1} vs. concentration of Cl^-, where Cl^- is direct proportonal to e^-_{aq} (see Figure 3.1).

2.3.1.4.2 MCAA Actinometry - Procedure (Curve B)

Next the intensity of the UV light must be specified by MCAA actinometry. The formation of $Fe(SCN)^{2+}$ is determined, which is directly related to the quantum yield of Cl^- ($Q(Cl^-)$) and thereby the intensity of the lamp can be calculated (equation (2.3)). Therefore a 2 M MCAA solution was prepared and irradiated. A concentration of 2 M was chosen to achieve a complete absorption at the given thickness of the solution (d = 1 cm), at least OD = 2 (equations (2.4) and (2.5)) [49, 46].

Lambert-Beer law:

$$\begin{aligned} OD &= \varepsilon \times d \times c \\ \varepsilon &= 2\ M^{-1}cm^{-1} \end{aligned} \tag{2.4}$$

$\varepsilon_{253.7}$ M^{-1} cm^{-1} is determined via the absorption spectrum of aqueous MCAA solution, see figure 2.2 .

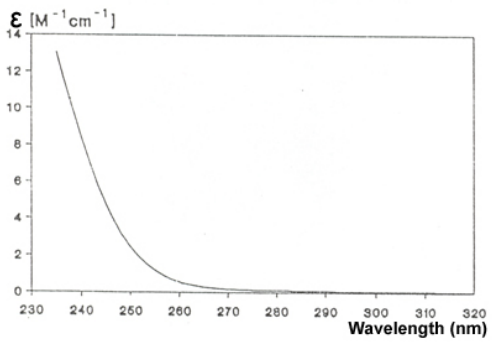


Figure 2.2 – Absorbtion spectrum of aqueous MCAA solution. ε_{254} = 2 M^{-1} cm^{-1}.

$$\begin{aligned} c = \frac{OD}{\varepsilon \times d} = \frac{2}{2M^{-1}cm^{-1} \times 1cm} \\ c = 2M \end{aligned} \tag{2.5}$$

MCAA actinometry:

- Prepare 200 ml of a 2 M L^{-1} MCAA solution and pour it into the irradiation vessel.
- Stir for about 20 min at 37° C using a thermostat.
- Put 0.2 ml of the unirradiated solution into a 10 ml flask. Add 1 ml of solution B and then one ml of solution A. Fill up to ten ml with 3xdH_2O. Mix well. This is the Ø value.
- Wait for 5 minutes and measure OD_{460} with the spectrophotometer. Use 3xdH_2O as reference.
- Start irradiation of the solution with UV light. Take samples of 0.2 ml after 1, 2, 3, 4, 5 and 6 minutes and prepare them the same way as the Ø value.
- Calculate ΔOD_{460} of each of the samples by substracting the OD_{460} of the Ø value from the OD_{460} of the different samples.
- Create a diagram: UV irradiation time versus ΔOD_{460}.
- Calculate slope of the curve and I_0 using equation (2.6).
- Considering the different dilution factors throughout the procedure (calibration line 1 ml to 10 ml, irradiation curve 0.2 ml to 10 ml) the result of the calculation above has to be multiplied by factor 50.

$$I_0 = k \times \frac{N_L}{Q(Cl^-) \times 1000} \tag{2.6}$$

k	=	slope of the curve
L	=	Loschmidt constant (6.022×10^{23} molecules mol^{-1})
$Q(Cl^-)$	=	quantum yield of Cl^-, depending on temperature

2.3.2 γ-Ray Irradiation

The experiments in vitro were performed under specific conditions by treatment with γ-ray, provided by a ^{60}Co source (Gammacell 220).

2.3.2.1 Gammacell 220

The Gammacell 220, AECL, Nordion Ltd., Canada, was used to maintain irradiation experiments in vitro. Details are given in Figure 2.3.

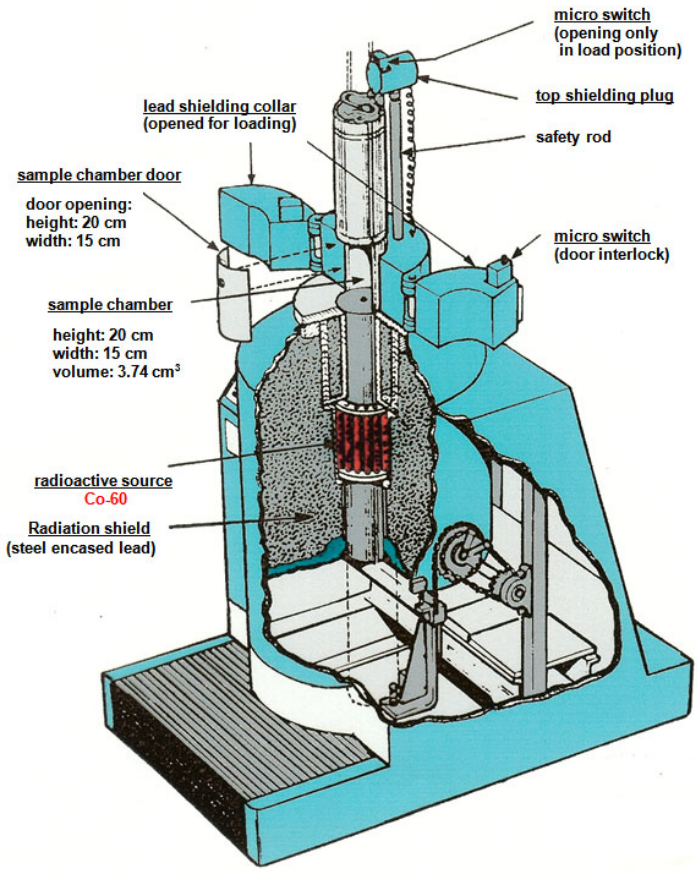


Figure 2.3 – The ^{60}Co γ-source, Gammacell 220, drawn in load position. Figure was kindly provided by Ass. Prof. Dr. Ruth Quint.

2.3.2.1.1 ^{60}Co-Isotope

^{60}Co is formed through neutron irradiation within atomreactors, according to reaction (2.7)) and (2.8) [50, 51].

$$^{59}_{27}Co + n \longrightarrow {}^{60}_{27}Co + \gamma \tag{2.7}$$

$$^{60}_{27}Co \longrightarrow {}^{60}_{28}Ni + e^{-} + \bar{\nu}_e + \gamma \tag{2.8}$$

^{60}Co, with a half-life of 5.2714 years, is the most used γ-ray source for various purposes. The primary decay mode of ^{60}Co is beta-decay. One β-particle with an energy level of 0.31 MeV is emitted, resulting in an exited $^{60}_{28}$Ni as decay product.

The resulting $^{60}_{28}$Ni* instantaneously emits two γ-quanta, with 1.17 MeV and 1.33 MeV, leading to stable $^{60}_{28}$Ni, as seen in equation (2.8) and figure 2.4.

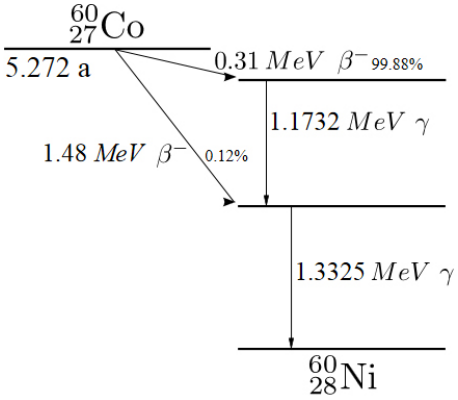


Figure 2.4 – ^{60}Co-decay scheme. Source:"Cobalt-60 Decay Schemep.svg", http://de.wikipedia.org/wiki/Cobalt, 2013-08-24.

2.3.2.2 Fricke Dosimetry

As the activity of a radioactive source is steadily decreasing according to its half-life, the actual activity of the source was calculated by "Kobaltquelle Gammacell 220" software, which is recalculating the acitvities monthly to ensure application of the right dose. The program is based on the determination of the activity by Fricke Dosimetry, which is a ferrous sulphate dosimetry using the oxidation of Fe^{2+} to Fe^{3+} in aqueous sulphuric acid solution under irradiation. This reaction could be followed by spectrophotometric measurement at a wavelength of 303 nm.

Dosimetric procedure according to the manual "Dosimetry for Food Irradiation" of the IAEA [52]:

- Prepare Fricke solution: 0.392 g $Fe(NH_4)_2(SO_4)_2 \cdot 6\,H_2O$ + 0.058 g NaCl in 1 L of H_2SO_4 (= 0.4 mol L^{-1} sulphuric acid, 0.001 M ferrous ammonium sulphate and 0.001 M sodium chloride).
- Fill irradiation containers for samples of Ø, 40, 80, 120, 160 and 240 seconds of irradiation and irradiate them for the defined time.
- The Ø value is determined by giving it into the loading chamber, lowering the drawer followed by immediate elevation.
- Determine OD at 303 nm using non irradiated Fricke solution as reference.

- Determine the temperature of the solution and calculate the molar extinction coefficient according to Figure 2.5.
- Calculate the absorbed dose of each irradiated Fricke dosimetric solution using equation (2.9).

$$D = \frac{(\Delta A)N_A}{p.G.\varepsilon.d} \tag{2.9}$$

where

D = absorbed dose in Gray (Gy), 1 Gy = 100 rad 6.24×10^{15}eV/g,
ΔA = the change in absorbance at 303 nm between irradiated (A_i) and not irradiated (A_0) samples, $\Delta A = A_i - A_0$, at 25° C,
N_A = Avogadro's number ($6.022 \times 10^{23} mol^{-1}$),
p = density of the dosimetric solution ($1.024 \times 10^3 kg/m^3$),
G = radiation chemical yield of Fe^{3+} ions (9.74×10^{17}molecules J^{-1}),
ε = molar absorption coefficient at 303 nm and 25° C (should be at a nominal value of 219 m^2 mol^{-1} in a good optical system [52]) and
d = optical path length (1 cm).

- using the given values of G and ε, reduces equation (2.9) to:

$$D_{Fricke}(Gy) = 278\ \Delta A \tag{2.10}$$

- Draw a diagram illustrating irradiation duration versus D (Gy).
- Determine the slope of the curve.

2.4 Saturation of solutions with Air, Ar or N_2

To obtain particular irradiation environments leading to specific formation and concentrations of radicals, the solutions where either saturated with air, Ar or N_2. In this way, reaction mechanisms could be manipulated in a more oxidative or reductive direction, see Table 2.4. Oxygen acts as a strong e^-_{aq} scavenger, drastically influencing chemical reactions because of $O_2^{\bullet -}$ formation. For UV irradiation purposes solutions were saturated directly within the irradiation vessel

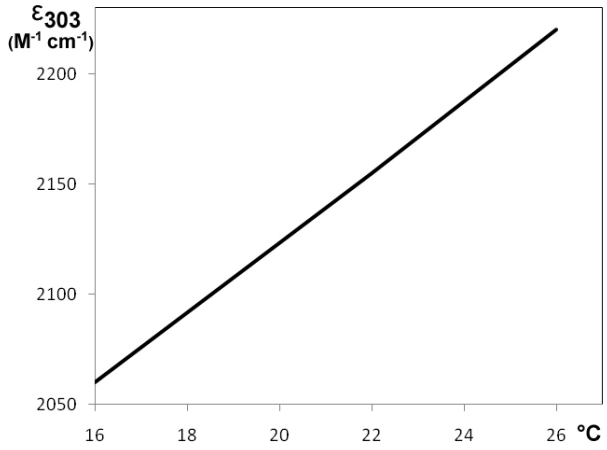


Figure 2.5 – Molar extinction coefficients of Fe(III) ions (ε in mol L^{-1} cm^{-1}) as a function of temperature (in °C) [53].

before irradiation for about 20 minutes. Therefore, the 4π-irradiation vessel provides a gas in- and outlet, allowing steady saturation of the solutions with different gases, while stirring with a magnetic bead (see Figure 2.1). Another reason for saturating solutions with Ar or N_2 is to prevent soluted substances from unwanted oxidation and degradation.

Procedure:

- Fill 3xdH_2O or sample in a flask or testtube.
- Cover it neatly with parafilm. When saturating with air, leave flask or add substances.
- For saturating the solution with Ar or N_2, penetrate the parafilm with a Pasteur pipette.
- Connect the gas hose to the pasteur pipette and saturate the liquid for at least 20 minutes.
- Unplug gas hose and remove the pasteur pipette, sealing the flask instantly with parafilm.
- Further proceedings according to the following protocols.

Table 2.4 – Water radiolysis. Formation of primary products and their yields (G-values) in pH-range 6.0 to 8.6. Source: Nikola Getoff, Free radicals effect on cytostatica, vitamins, hormones and phytocompounds with respect to cancer. [54]

$$
\begin{array}{lcl}
H_2O \rightsquigarrow H_2O^* & \longrightarrow & H + OH \\
 & \longrightarrow & H_2O + e_s^- \\
e^- & \longrightarrow & e_{th}^- + nH_2O \longrightarrow e_{aq}^-
\end{array}
$$

$$
\begin{array}{lcl}
H_2O^+ + H_2O & \longrightarrow & H_3O^+(H_{aq}^+) + OH \\
e_{aq}^- + OH & \longrightarrow & OH_{aq}^- \\
e_{aq}^- + H_a^+ q & \longrightarrow & H
\end{array}
$$

Primary products of water radiolysis:

$H_2O \rightsquigarrow$	e_{aq}^-,	H,	OH,	H_2,	H_2O_2,	H_{aq}^+,	OH_{aq}^-
(G-value at pH 6-8.6)	(2.7)	(0.6)	(2.8)	(0.45)	(0.7)	(3.2)	(0.5)

In the presence of air H and e_{aq}^- are converted into peroxyl radicals:

$$
\begin{array}{lcll}
H + O_2 & \longrightarrow & HO_2^\bullet & (k = 2 \times 10^{10} L.mol^{-1}.s^{-1}) \\
e_{aq}^- + O_2 & \longrightarrow & O_2^{\bullet -} & (k = 1.9 \times 10^{10} L.mol^{-1}.s^{-1}) \\
HO_2^\bullet & \rightleftharpoons & H^+ + O_2^{\bullet -} & (pK = 4.8)
\end{array}
$$

By saturation of the aqueous solution with N_2O, the reducing e_{aq}^- are specifically transformed into oxidizing OH-radicals:

$$e_{aq}^- + N_2O \longrightarrow OH + OH^- + N_2 \quad (k = 0.91 \times 10^{10} L.mol^{-1}.s^{-1})$$

Therefore, the acting species in different media (pH = 6.4 to 8.6) are:

Airfree	Air	N_2O
$46\% OH$	$46\% OH$	$90\% OH$
$44\% e_{aq}^-$	$54\% O_2^\bullet -$	$10\% H$
$10\% H$		

G-value = number of produced or decomposed molecules per 100 eV absorbed energy.
For conversion into SI-units multiply the G-value by 0.10364 to obtain G(x) in µmol J^-.
1 Gy = 100 rad = 6.24 $\times 10^{15}$ eV g^{-1}.
1 Gy generates 6.344 $\times 10^{-7}$ mol L^{-1} (e_{aq}^- + H + OH).

2.5 Determination of Electron Emission

For determination of the e^-_{aq} yield ejected by CYC, VitC as well as their mix, solutions were irradiated with monochromatic UV-light at $\lambda = 254$ nm (E = 4.85 eV $h\nu^{-1}$; 1 eV = 23 kcal mol^{-1}). Chloroethanol (ClEtOH) was used as e^-_{aq} scavenger, as resulting e^-_{aq} , emitted from the substances during UV-irradiation, are leading to the formation of Cl^-. Chloroethanol itself is not excited by UV-light at a wavelength of $\lambda = 254$ nm and therefore used as Cl^- donor. The yield of e^-_{aq} is directly proportional to the resulting Cl^- yield, which is determined by spectrophotoscopy at $\lambda = 460$ nm.

2.5.1 Spectrophotometer

For spectrophotometric measurements a Perkin Elmer, Lambda 650 UV/VIS Spectrometer was used with quartz cuvettes, "HELLMA, Präzisions-Küvetten, Quarz Suprasil", TypeNo. 100 QS, with a light path of 10 mm. The Perkin Elmer UV WinLab 6.0.2.0723 as well as Perkin Elmer UV WinLab Data Processor and Viewer (DPV) 1.00.00.0006 Software were applied for data analysis and processing.

2.5.2 Preparation

Samples were prepared as indicated below:

- Pour 200 ml 3xdH_2O into the irradiation vessel (Figure 2.1).
- Stir for about 20 min at 37° C using a thermostat, while saturating the solution with Ar or N_2.
- Add the calculated amount of the substance to the Ar saturated water, keep saturating with Ar and stirring throughout the experiment. Pay attention when using CYC or other proteinogenic substances, because they will foam when being saturated with too much pressure.
- Wait some minutes to make sure that the substances are well dissolved.
- Measure pH and adjust to pH 7.4 using NaOH or $HClO_4$.
- Take 1 ml and make an absorption spectrum from 200 - 500 nm with the spectrophotometer. Use 3xdH_2O as reference.
- Determine OD_{254} of the solution. Put the used solution back to the vessel.

- Add 1×10^{-2} mol L^{-1} ClEtOH to the solution in the vessel (134 µl per 200 ml). Measure pH of the solution.
- Put 1 ml from the initial solution into a 10 ml flask. This will be the blank, Ø value. Add firstly 1 ml of reagent B and then 1 ml reagent A (preperation see at chapter 2.3.1.4.1). Fill up to 10 ml with 3xdH_2O and mix well. Wait for 5 min and determine OD_{460}.
- Start irradiation of the solution with UV light. Take samples of 1 ml after given timepoints (see table 2.5) and prepare them the same way as the Ø value.
- After UV-irradiation wait for 5 minutes and measure pH. Make an absorption spectrum from 200 - 500 nm as well.
- Calculate ΔOD_{460} of each of the samples by substracting the OD_{460} from the Ø value from the OD_{460} of the different samples using the template shown in figure 2.6.

Table 2.5 – Timepoints for sampling for quantum yield determination. Two different sampling frequencies were used to cover the most important areas of e^-_{aq} development during UV irradiation.

Time in minutes							
0	2	4	6	9	12	15	18
and							
Time in							
seconds			**minutes**				
0	20	60	4	9	14	18	

2.5.2.1 Determination of e^-_{aq} Emission

To determine the yield of e^-_{aq} (mol L^{-1}) the ΔOD_{460} has to be calculated, like already mentioned above. Using the NaCl calibration curve (Figure 3.1) the e^-_{aq} (mol L^{-1}) yield is defineable by directly picking the concentration of e^-_{aq} from the diagram. Therefore, the calculated ΔOD_{460} can be directly read from the $(\frac{OD}{cm})_{460}$ axis, resulting in the e^-_{aq} value by crossing the calibration line and reading out the value at this point. This way specified values are now inserted into the template 2.6.

Experiment 2 with Cytochrome c, 200 ml Vessel, 20100902

Concentration: 5 x 10^{-6} mol L^{-1} bovine cytochrome c
Amount: 200 ml in H_2O, Ar saturated

OD cm^{-1} (254 nm):

1 x 10^{-6} mol L^{-1} **pre UV:** 0.015061 **post UV:** 0.01257

2.5 x 10^{-6} mol L^{-1} **pre UV:** 0.04389 **post UV:** 0.042548

5 x 10^{-6} mol L^{-1} **pre UV:** 0.08631 **post UV:** 0.09221

OD cm^{-1} (407 nm):

1 x 10^{-6} mol L^{-1} **pre UV:** 0.095993 **post UV:** 0.067533

2.5 x 10^{-6} mol L^{-1} **pre UV:** 0.23192 **post UV:** 0.17218

5 x 10^{-6} mol L^{-1} **pre UV:** 0.46197 **post UV:** 0.3382

Temperature: 37° C

pH (0 min) 5.8

pH (6 min) 5.4

pH (15 min) 5.2

$OD = \varepsilon \times c \times d$

$\varepsilon = L\ mol^{-1}\ cm^{-1}$

$I_A = I_0 - I = 1 \times 10^{18} - 10^{17.91369} = 1.8 \times 10^{17}$ hv $ml^{-1}\ min^{-1} = 0.18 \times 10^{18}$ hv $ml^{-1}\ min^{-1}$

time	OD cm^{-1} λ = 460 nm	Δ OD cm^{-1} λ = 460 nm	e^-_{aq} (mol L^{-1})	I_{abs} (hv L^{-1})
1.) 0 min	0.0142	0	0	0
2.) 2 min	0.0196	0.0054	2.8 x 10^{-6}	0.36 x 10^{21}
3.) 4 min	0.0196	0.0054	2.8 x 10^{-6}	0.72 x 10^{21}
4.) 6 min	0.0163	0.0021	1.1 x 10^{-6}	1.08 x 10^{21}
5.) 9 min	0.0183	0.0041	2.1 x 10^{-6}	1.62 x 10^{21}
6.) 12 min	0.0156	0.0014	0.8 x 10^{-6}	2.16 x 10^{21}
7.) 15 min	0.0151	0.0009	0.5 x 10^{-6}	2.70 x 10^{21}
8.) 18 min	0.0191	0.0049	2.5 x 10^{-6}	3.24 x 10^{21}

Figure 2.6 – Template for determination of e^- yield. This template was used, with slight adaptions, throughout all UV irradiation experiments for determination of the quantum yields of the different substances. It shows all the important data, from OD_{460} to ΔOD_{460} as well as the calculated e^-_{aq} and I_{abs} needed for later calculations and establishment of a diagram showing the e^-_{aq} emission as a function of absorbed UV dose as well as for HPLC measurements.

Additionally, the I_{abs} ($h\nu$ L^{-1}) must be calculated, to define the energy input per developed e^-_{aq} by using the equation:

$$I_A = I_0 - I \tag{2.11}$$

Whereby OD = $\log(I_0/I)$, I_0 is the determined intensity of the UV lamp (see 2.3.1.4.2) and I is the OD_{254} picked from the absorption spectrum taken at the beginning.

Using this information it is possible to construct a diagram showing e^-_{aq} mol L^{-1} in relation to $h\nu$ L^{-1} (see figure 3.4).

2.6 High Performance Liquid Chromatography

Analyses of the final products formed by photolysis of CYC and VitC as well as their mixtures were performed by High Performance Liquid Chromatography (HPLC) method. Following this pathway, also the regenerative activity of VitC, forming metabolites by e^-_{aq} transfer, could be determined. HPLC is a commonly used analytical method. It allows separation of single chemicals within a mixture due to their different flow behaviour in dependence of their mobile phases and seperation columns used. The behaviour of CYC and VitC as well as their mixture under the impact of UV irradiation was analysed by HPLC. Degradation of the substances and the potential formation of metabolites was surveyed.

HPLC was maintained by a Hewlett Packard Series 1100 combined with a 1050 diode array detector and an Agilent Poroshell 300 SB-C8, 2.1 x 75 mm, 5 µm particle size, column. Data were analysed with ChemStation for LC 3D, Rev.A.10.02 [1757], Software from AGILENT Technologies. Amber glassed screw-top HPLC vials with wide opening, prod.nr 5182-0716, were ordered from AGILENT Technologies.

2.6.1 Settings

As already mentioned, a C8 column was used for anlysis of the samples. As CYC is a quite large molecule with about 13 kDa, C3 or C4 columns are usually used for

HPLC analysis of CYC solutions [55]. VitC and hormone derivates are typically measured with a C18 column, due to their small size. It was not possible to get a strong and fast signal using the C18 column for CYC analysis, as well as VitC was hard to detect with a C3 or C4 column. Therefore, the C8 column was chosen to get good results and strong peaks of mixtures with CYC and VitC.

20 µl of each sample were injected and eluted at 30° C by a linear gradient of mobile phase (A) to mobile phase (B). The composition of the two phases are given at table 2.6. The gradient was started with 100 % of phase (A) and 0 % of (B) decreasing to 0 % of (A) and increasing to 100 % of (B) within 5 minutes, held for 2 more minutes. The flow rate was set to one ml per minute. Detection of CYC was performed at 407, 409 and 414 nm and of VitC at 250 nm.

Table 2.6 – Mobile Phases for HPLC analysis

Mobile Phase (A)	**Mobile Phase (B)**
0.1 % Formic acid in dH_2O	0.1 % Formic acid in Acetonitril

2.6.2 Sample Preparation

The samples were prepared similar way as to the one used for determination of e^-_{aq} , see 2.5.2, but no other substances were added to the samples and the sampling frequency was higher compared to that of e^-_{aq} measurement. The timepoints chosen are given in Table 2.7.

Preparation:

- Pour 200 ml 3xdH_2O into the irradiation vessel.
- Stir for about 20 min at 37° C using a thermostat, while saturating the solution with Ar or N_2.
- Purge the HPLC vials with Ar or N_2 to minimize oxidation of the samples afterwards.
- Add the calculated amount of the substance to the Ar saturated water, keep saturating with Ar and stirring throughout the experiment. Pay attention when using CYC or other proteinogenic substances, because they will foam

a lot when saturating with too much pressure.

- Wait some minutes to make sure that the substances are well dispensed.
- Measure pH and adjust to pH 7.4 using NaOH or $HClO_4$.
- Take 1 ml and make an absorption spectrum from 200 - 500 nm with the spectrophotometer. Use the 3xdH_2O as reference.
- Determine OD_{254} of the solution. Put the used solution back to the vessel.
- Put 1 ml from the initial solution into a HPLC vial, cover with Ar or N_2 and close properly. This will be the Ø value.
- Start irradiation of the solution with UV light. Take samples of 1 ml after given timepoints (see table 2.7) and prepare as described above.
- After irradiation wait for 5 minutes and measure pH. Make an absorption spectrum from 200 - 500 nm as well.
- Calculate ΔOD_{460} of each of the samples by substracting their OD_{460} from the Ø value.
- Determine the number of absorbed quanta, whereby OD = log (I_0/I) and I_A = I_0 - I.
- Start HPLC apparatus and load the program with the gradient and detection wavelength as described above.
- Load the samples into the HPLC apparatus and start program.
- If more runs are done in series, it is favorable to run a 3xdH_2O sample in between to rinse the column.
- Print the HPLC chromatogrammes and compare the areas (mAU) of the specific peaks.

Table 2.7 – Timepoints for sampling

Time									
seconds							minutes		
0	20	40	60	90	120	150	4	6	9

2.7 Experiments In Vitro with MCF-7 cells

The MCF-7 cancer cell line is well explored and frequently applied for cancer studies. It derives from a human metastatic mammary adenocarcinoma and was established from a 69 year old woman after radiation and hormone therapy in

1970 [56]. These epithelial cells are able to form domes and to synthesize estradiol. Due to the presence of variant estrogen receptors as well as a progesterone receptor this cell line was applied for the experimental work with sex hormones [57, 58, 59, 60]. MCF-7 cells were chosen as a model for the cell culture experiments.

The MCF-7 cell line was kindly provided by Ao.Univ.-Prof. Mag. Dr. Christian Schneeberger, Departement for Gynecological Endocrinology and Reprocuctive Medicine, Medical University of Vienna and Maria Eisenbauer, Institute of Cancer Research, Medical University of Vienna.

2.7.1 Microscope

A NIKON TMS-F, with NIKON lenses of 4, 10, 20, and 40 fold magnification was used for all microscopic work. Cells were usually microscoped at a 100 fold magnification.

2.7.2 Counting Chamber

A BRAND, Bürker-Türk counting chamber, depth 0.100 mm, 0.0025 mm^2, 0.04 mm^2, at 100 fold magnification was used for determination of cell numbers.

2.7.3 Cultivation of MCF-7 cells

For culturing MCF-7 cells following materials were used:

Table 2.8 – Working materials for cell culture.

Materials	Prod.No.	Company
10 ml one way pipettes	47110	Sterilin
12 Well Culture Plate	665180	greiner bio-one
15 ml Falcons	15CTRP	Sterilin
Easy Flask, 25 V/C	156340	NUNC
Easy Flask, 175 V/C	159920	NUNC
Multi-Reactiontubes, 1.7 ml	7080.1	ROTH
Needle, BD Microlance 3, 0.8 mm x 40 mm	304432	BD Biosciences
Sterile Syringe Filter, 0.2 µm, cellulose acetate	514-0061	VWR
Sterile Syringe, Discardit II, 10 ml, luer	613-3952	BD Biosciences

The buffers for cell culture were bought at following specifications:

DMEM 1x:

+ 1 g/L D-Glc
+ Pyruvate
- L-Glutamin
- Phenolred

PBS pH 7.4, 10x:

- $CaCl_2$
- $MgCl_2$

The cells were incubated in a Cytoperm, Heraeus Instruments Incubator, at 37° C and a humidified atmosphere containing 5 % CO_2.

For centrifugation steps a Heraeus Sepatech Megafuge 1.0, with a BS4402/A Her.Sep., Cat.-No. 3360 rotor, was used. For propagation MCF-7 were cultivated in an "Easy Flask, 175 V/C", while cells for experiments where either cultivated in an "Easy Flask, 25 V/C" or a 12-well culture plate (for toxicity tests).

2.7.3.1 Culture Medium

Cell culture medium was prepared according to ATCC [61] or DSMZ [62] specifications with slight adaptions.

Cell Culture Media:

			550 ml:		
90	%	DMEM	500	ml	DMEM
10	%	FCS	55	ml	FCS
10	µg/ml	bovine insuline	31.35	µl	Insulin from stock (see below)
2	mM L^{-1}	L-Gln	161	mg	L-Gln
1.8	mM L^{-1}	D-Glc	1.65	g	D-Glc

Dissolve L-Gln and D-Glc in some of the DMEM and sterile filtrate the solution directly to the rest of the DMEM. Add FCS and the insulin from the stock and mix well. Store at 4° C and warm at 37° C right before use.

Insulin Stock Solution:

2×10^{-3}	M L^{-1}	Insulin	210	mg	Insulin
			in 4320	µl	0.1 M HCl solution

Dissolve insulin in HCl while stirring.

2×10^{-5}	M L^{-1}	BSA	24.6	mg	BSA
0.9	%	NaCl in dH_2O	20	ml	NaCl solution

Add 15.7 ml of the BSA solution to the insulin solution while stirring. Sterile filtrate the mixture. Store at 4° C.

2.7.3.2 Splitting and Seeding

Propagated cell culture had to be split every 3 days to ensure their staying in exponential growth phase.

Procedure:

- Temper cell culture media and PBS at 37° C within a waterbath.
- Discard supernatant from the flask.
- Wash with 10 ml PBS if Easy Flask, 175 V/C is used, in case of Easy Flask, 25 V/C use 3 - 4 ml PBS.
- Discard PBS and add 1.5 ml Trypsin/EDTA to the flask (about 500 µl for 25 V/C).
- Incubate for 3 - 5 minutes at 37° C in the incubator till all cells are detached from the surface. Do not agitate too much as MCF-7 cells tend to aggregate easily.
- Add 10 ml of culture medium (1 ml for 25 V/C) to deactivate the trypsin and pipette suspension into a petri dish (ø = 9 cm).
- Shear cells by drawing the suspension through a sterile needle (ø = 0.8 mm) with a syringe.
- If cells are just split for propagation, split into thirds, seed in three 175 V/C flasks and add 15 ml of culture media.
- Otherwise determine cell number per ml and seed the needed cell number in 25 V/C flasks or wells of 12-well plate.

- Count the cells with the counting chamber. Summarize the cell numbers per counting array. Divide through enumerated fields and multiply by 10^4 (according to the chamber depth and area of the counting arrays).
- Knowing the cell number per ml, seed about 300.000 cells per 25 V/C flask or about 30.000 cells per well of a 12-well plate.
- Add 5 ml of culture medium to a 25 V/C flask or 2 ml per well of a 12-well plate.
- Put at 37° C into the incubator an let them grow for at least one day before further treatment.

2.7.4 Treatment with Cytochrome c and Ascorbic Acid

2.7.4.1 Stock Solutions

For chemical treatment of the cell cultures solution stocks were prepared.

1 x 10^{-3} M L^{-1} Cytochrome c Stock Solution:

			10 ml:		
0.001	M	CYC	123.2	mg	CYC
		oxygen free 3xdH_2O	10	ml	oxygen free 3xdH_2O

Quickly dissolve CYC in oxygen free 3xdH_2O. Do not mix too roughly to prevent solution from foaming and getting too high oxygen yields. Sterile filtrate and cover with Ar or N_2 via filter and sterilized gas hose or use instantly.

10 ml of a 1 x 10^{-3} M L^{-1} CYC (MW 12.327 g mol^{-1}) stock solution were prepared. When handled properly, lightprotected, covered with Ar or N_2 to prevend oxidation and at 4° C, CYC is just slowly degraded and stable for up to one week.

1 x 10^{-1} M L^{-1} Ascorbic Acid Stock Solution:

			5 ml:		
0.1	M	VitC	88	mg	VitC
		oxygen free 3xdH_2O	5	ml	oxygen free 3xdH_2O

Quickly dissolve VitC in oxygen free 3xdH_2O. Do not stir roughly to prevent solution from getting too high oxygen yields. Sterile filtrate and cover with Ar or N_2 via filter and sterilized gas hose or use instantly.

For experiments using VitC (MW 176.12 g mol^{-1}) 5 ml of a 1 x 10^{-1} mol L^{-1} VitC stock solution was prepared. This stock was then diluted to concentrations of 1 x 10^{-2} mol L^{-1} and 1 x 10^{-3} mol L^{-1} VitC for the use in vitro, depending on needed concentrations and amount to be supplemented. Since VitC is not very stable in aqueous solution and gets degraded quite quickly, the stock solution was prepared freshly every day needed.

2.7.4.2 Toxicity Test

To ensure that recognized effects derive just from irradition treatment and not because of other effects of the substances, toxicity tests were performed. This was of special interest in case of CYC, because of its known role in apoptosis, where it is leading to cell death, when located in the cytoplasm.

Table 2.13 – Concentrations used for toxicity tests on MCF-7 cells. Table is showing the concentrations for CYC and VitC as only supplement as well as testet concentrations for mixtures of them.

solo:	**CYC** mol L^{-1}	**VitC** mol L^{-1}	**mix:**	**CYC** mol L^{-1}	+	**VitC** mol L^{-1}
	5×10^{-6}	7.5×10^{-7}		1×10^{-6}	+	1×10^{-6}
	1×10^{-5}	1×10^{-6}		2.5×10^{-6}	+	2.5×10^{-6}
	5×10^{-5}	2.5×10^{-6}		5×10^{-6}	+	5×10^{-6}
	7.5×10^{-5}	5×10^{-6}		7.5×10^{-6}	+	7.5×10^{-6}
	1×10^{-4}	7.5×10^{-6}		1×10^{-5}	+	1×10^{-5}
	2.5×10^{-4}	1×10^{-5}		2.5×10^{-5}	+	2.5×10^{-5}
		5×10^{-5}				
		1×10^{-4}				
		5×10^{-4}				

Procedure:

- Seed cells in 12-well plates and grow for 24 hours.
- Discard culture medium and add 2 ml of fresh medium.
- Add substances from the stocks in the amount needed for the different concentrations chosen (see table 2.13) to the designated wells.
- Shake carefully to mix the substances with the media.
- Incubate for one hour, discard medium and add fresh medium and incubate for 24 hours with the substances.
- Determine cell growth and possible effects on the cells.

2.7.5 γ-Ray Irradiation of MCF-7 cells

For γ-ray experiments cells were cultivated within 25 V/C flasks until a density of about 30 - 40 percent. Treatment with the substances occurred about one hour before irradiation.

Procedure:

- Cultivate MCF-7 cells to a density of 30 to 40 %.
- Calculate needed amounts of substances from the stock.
- Discard supernatant properly.
- Add culture medium according to the needed amount of stock solution for the wanted concentration to get a total of 5 ml of culture medium. For untreated cells just add 5 ml of cell culture medium.
- Add substance from stock in the calculated amount.
- Shake carefully to mix the substances with the medium.
- After one hour irradiate the cells with the doses needed (doses at table 2.14).
- The Ø value is treated the same way as the irradiated cells, but not given into the γ-ray source.
- Put irradiated cells back into the incubator.
- Determine cell survival after two days, when the Ø value reaches a density of about 85 - 90 %.
- Draw survival curves and determine D_{37} value (dose were 37% of the cells survive).

2.7.5.1 Irradiation Dose

To apply the right dose of irradiation the duration of irradiation was calculated as explained in chapter 2.3.2. The duration depends on the actual activity of the γ-ray source and was determined monthly. Doses set are given in table 2.14 below.

Table 2.14 – Irradiation doses for experiments in vitro with MCF-7 cells.

Radiation Doses (Gy)						
0	5	10	15	20	25	30

2.7.5.2 Survival Counts

- Discard supernatant and wash well with 3 ml of PBS.
- Discard PBS and add 500 µl Trypsin/EDTA. Put for 3 - 5 min into the incubator.
- When all cells are detached, add 1 ml of culture medium to the flasks with denser population (0, 5 and 10 Gy) and 500 µl to the flasks with fewer density to deactivate the trypsin. This is important for later counting of the cells.
- Resuspend the cells well with a 1 ml pipette to obtain a single cell suspension.
- Transfer suspension into a 1.5 ml reaction-tube.
- Count with the counting chamber, resuspend again with a 200 µl pipette before loading the chamber.
- Calculate the cell number per flask recognizing the different dilution factors.
- Draw a survival curve N/N_0 ratio versus dose (Gy) and determine the D_{37} value.

2.7.5.3 Survival Curves and D_{37} value

The survival curve presents the number of surviving cells against the absorbed dose. The number of surviving cells is given in their ratio N/N_0, where the unirradiated sample is 1 (N_0) and the values of the irradiated samples are given by the ratio N/N_0 (see Figure 3.21).

The D_{37} value presents the dose where 37 % of the cells remain alive and can easily be picked from the survival curve. Comparison of the D_{37} values of the

differently treated cultures allows conclusion about a protective or non-protective property of the substances used. Therefore ΔD_{37} has to be calculated for each of the substances (equation 2.12).

$$\Delta D_{37} = D_{37(I)} - D_{37(0)} \tag{2.12}$$

were
$D_{37(I)}$ = D_{37} value of the substance treated culture and
$D_{37(0)}$ = D_{37} value of the culture irradiated without substance.

A positive ΔD_{37} is indicating that the substance has a protective effect against irradiation, whereas negative ΔD_{37} shows a destructive effect of the substance combined with irradiation. Protective effects are often derived from the ability of the substance of scavenging reactive molecule species, preventing cells being damaged by them. Destructive, enhancing effects might occur by the development of toxic degradation products affecting metabolism of the cells or in the formation of additional reactive species, like radical formation, supporting the effects of the hydrolysis products leading to higher cell death rates.

Chapter 3

Results

3.1 Aktinometry

To determine the I_0 of the UV-source, monochloracetic acid (MCAA) actinometry measurements were carried out [49, 46, 47]. The intensity I_0 of the UV lamp was calculated using equation (2.6) and the established NaCl calibration curve (Figure 3.1) as well as the MCAA curve (Figure 3.2). Additionally, the dilution factor of 50 has to be included.

$$\begin{gathered} I_0 = (2.3 \times 10^{-5}/2) \times (6.023 \times 10^{23}/0.42 \times 10^3) \times 50 \\ \mathrm{I_0} = 0.82 \times 10^{18} \mathrm{h}\nu.\ \mathrm{ml}^{-1}\ \mathrm{min}^{-1} \end{gathered} \tag{3.1}$$

3.2 Determination of e^-_{aq} Emission

3.2.1 Cytochrome c; e^-_{aq} yields

3.2.1.1 Molar extinction coefficient

By increasing the concentration of CYC in aqueous solution, the molar extinction coefficient is decreasing (Figure 3.3) and is not obeying Lamgert-Beer's law (2.4) [63]. This demonstrates that at higher concentrations associates (unstable complexes) of CYC are developed, as already known for several other organic compounds, such as hormones. It is important to recognize this effect, due to the fact

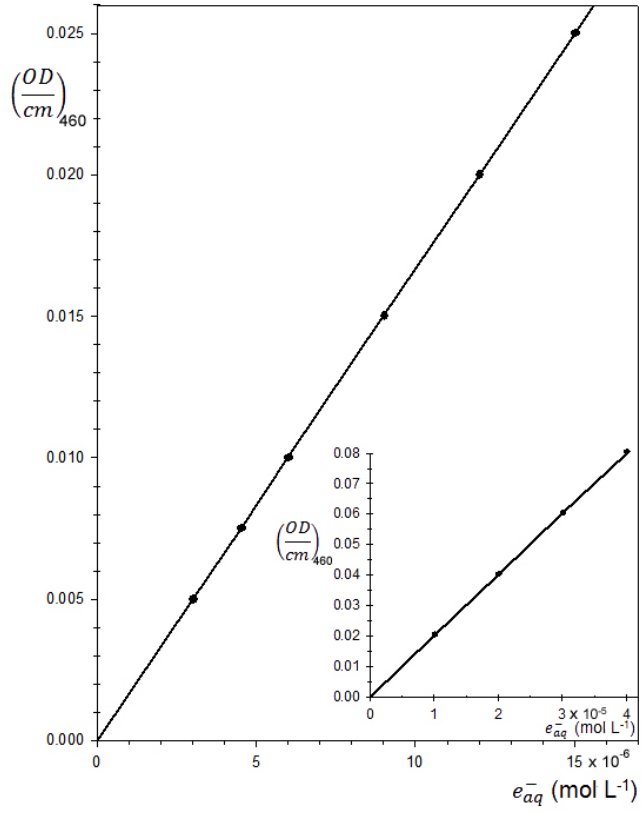


Figure 3.1 – NaCl calibration curve. The diagram shows OD/cm at $\lambda = 460$ nm versus concentration of Cl^- in mol L^{-1}.

that aggregated substrates do not show the same behavior under irradiation as their well dissociated counterparts. Associates are consuming a part of the emitted e^-_{aq} within their complexes, meaning that measured e^-_{aq} yields at higher concentrations do not correlate directly with e^-_{aq} yields of lower concentrations of CYC.

3.2.1.2 e^-_{aq} yields of CYC

Being irradiated with monochromatic UV light at 254 nm CYC is emitting e^-_{aq} by excitation in singlet state, as expected. CYC concentrations irradiated and photospectroscopically measured were:

(i) 1×10^{-5} mol L^{-1} and
(ii) 5×10^{-6} mol L^{-1}.

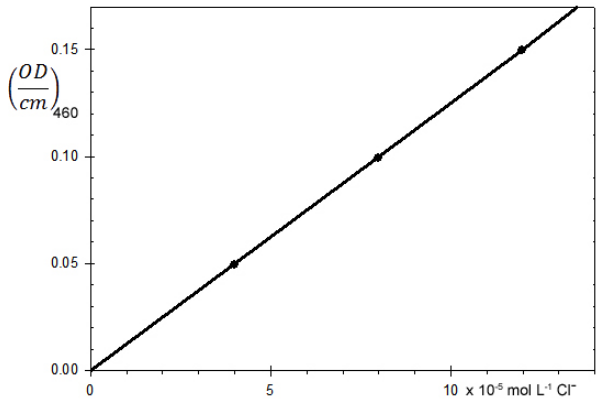


Figure 3.2 – Monochloracetic acid actinometry. Determination of I_0 of the UV-light source. Cl^- (mol L^{-1}) versus irradiation time in minutes. Cl^- correlates directly with e^-_{aq} , $Cl^- = e^-_{aq}$

It was intended to have a closer look at higher concentrations as well in the beginning, but because of the associate formation described in chapter 3.2.1.1, this would not have been reasonable.

The tested concentrations are showing a quite large first peak of e^-_{aq} emission, followed by a second, and in case of 1×10^{-5} mol L^{-1} CYC an additional delayed third peak of emitted e^-_{aq} as shown in Figure 3.4. The figure represents the data of the concentration of 1×10^{-5} mol L^{-1} CYC in A and of 5×10^{-6} mol L^{-1} CYC in the inset B. The diagrams are showing the emitted e^-_{aq} in mol L^{-1} as a function of absorbed UV-quanta in $h\nu$ L^{-1}. The initial quantum yields ($Q(e^-_{aq})_i$) are given in table 3.1 and 3.2 as well as in the insets of figure 3.4. The $Q(e^-_{aq})_i$ is calculated from the linear part of a curve, which occurs shortly after the irradiation start and originate from the substrate itself depending on the concentration, the solvent, temperature and possible reaction partners in mixes.

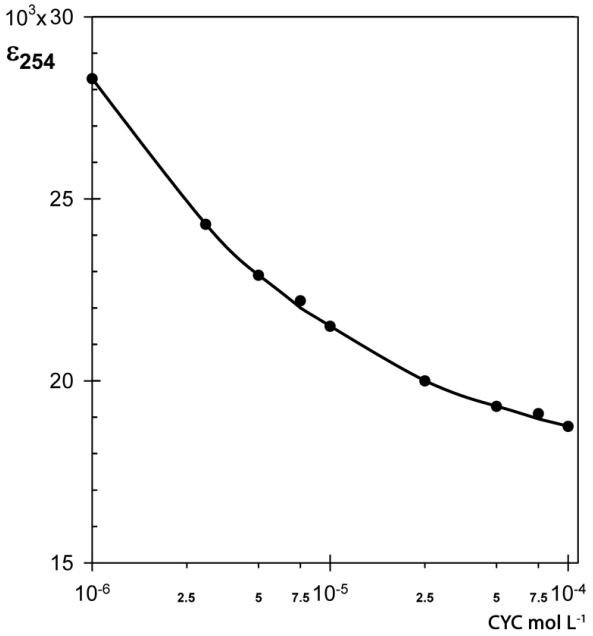


Figure 3.3 – Molar extinction coefficient (ε_{254}; L mol^{-1} cm^{-1}) in dependence of CYC concentration (mol L^{-1}), (pH = 7.4).

Table 3.1 – 1×10^{-5} mol L^{-1} CYC:

Peak	$Q(e^-_{aq})_i$
(A1)	$= 0.0115$ hν L^{-1}
(A2)	$= 0.0010$ hν L^{-1}
(A3)	$= 0.0004$ hν L^{-1}

Table 3.2 – 5×10^{-6} mol L^{-1} CYC:

Peak	$Q(e^-_{aq})_i$
(B1)	$= 0.0186$ hν L^{-1}
(B2)	$= 0.0041$ hν L^{-1}

At higher CYC concentrations e^-_{aq} emission occurs at a slightly lower $Q(e^-_{aq})_i$ than at the lower concentration, $Q(e^-_{aq})_i = 0.0115$ hν L^{-1} vs $Q(e^-_{aq})_i = 0.0186$ hν L^{-1}. The first peak is very sharp while the following are more stretched and at lower e^-_{aq} yields. At CYC $= 5 \times 10^{-6}$ mol L^{-1} just two peaks are recognized. At this low concentration CYC is nearly depleted after 3×10^{21} hν L^{-1}, so that no further e^-_{aq} emission is possible. Secondary and tertiary peaks are indicating e^-_{aq} emission originating from metabolites, but at significantly lower yields.

As expected, at higher concentrations the e^-_{aq} yield is lower compared to the e^-_{aq} yield of the lower concentration, due to the formation of associates, as shown in Tables 3.3 and 3.4.

e^-_{aq} yields (mol L^{-1}):

Table 3.3 – 1×10^{-5} mol L^{-1} CYC:

Peak	e^-_{aq}
(A1)	$= 5.76 \times 10^{-6}$ mol L^{-1}
(A2)	$= 3.30 \times 10^{-6}$ mol L^{-1}
(A3)	$= 3.16 \times 10^{-6}$ mol L^{-1}

Table 3.4 – 5×10^{-6} mol L^{-1} CYC:

Peak	e^-_{aq}
(B1)	$= 3.15 \times 10^{-6}$ mol L^{-1}
(B2)	$= 2.15 \times 10^{-6}$ mol L^{-1}

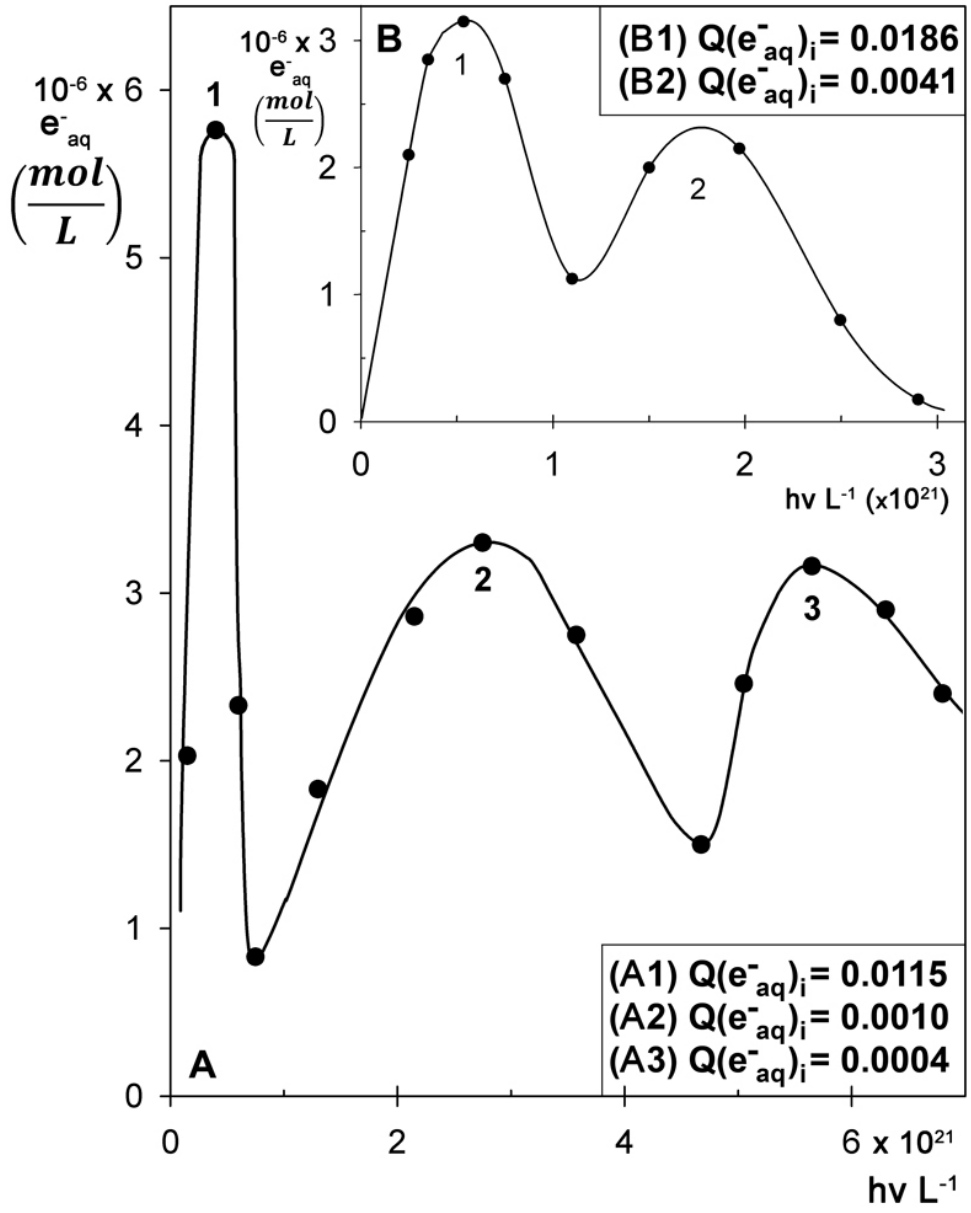


Figure 3.4 – Electron emission of airfree aqueous solution (pH $\approx$ 7.4) of CYC as a function of absorbed UV dose (hν L^{-1}). (A) 5×10^{-5} mol L^{-1}; (B) 1×10^{6} mol L^{-1} CYC. Insets are showing the quantum yields, $Q(e^{-}_{aq})_i$, at the associated peaks.

3.3 HPLC Analysis

Photolysis and production of metabolites were analysed by HPLC method. Samples were analysed at 407, 409, and 414 nm concerning CYC and at 250 nm concerning VitC. For calculations the ratios

$$\frac{mAU\%}{(mAU\%)_0} \tag{3.2}$$

where
mAU% is the area of the irradiated samples and
$(mAU\%)_0$ is the area of the unirradiated sample at timepoint Ø,

are given in percent.

3.3.1 Cytochrome c

CYC was analysed after UV-treatment in airfree as well as aerated solution. To obtain an airfree environment the solution was saturated with Ar directly within the irradiation apparature. Concentrations of CYC tested were: 5×10^{-6} mol L^{-1}, 1×10^{-5} mol L^{-1} and 5×10^{-5} mol L^{-1} in case of airfree tests. In aerated solutions 5×10^{-6} mol L^{-1} and 1×10^{-5} mol L^{-1} were surveyed. CYC shows a peak at rt = 2.66 min.

3.3.1.1 Photolysis of CYC - in airfree media

The photolytic effect of UV-irradiation on CYC in airfree solutions was determined by using data deriving from the 407 nm line. It was observed that the peak at the range between 407 nm and 414 nm is migrating from 407 towards 414 nm, indicating that a peak at 407 nm derives from reduced CYC, while the 414 nm peak stands for the oxidized form of CYC. Figure 3.5 represents the degradation of CYC as a function of absorbed UV-quanta. Lower concentrations show a faster photolysis compared to higher concentrations. The initial e^-_{aq} quantum yields of CYC and its different concentrations are given in table 3.5. The figures 3.6, 3.7 and 3.8 are presenting the HPLC chromatograms. The inital pointed peak is widening during irradiation time and the formation of two additional peaks is

observed, indicating metabolite formation.

Table 3.5 – Initial quantum yields of photolysis.

Curve	CYC	$Q(CYC)_i$
(A)	5×10^{-6} mol L^{-1}	0.0003
(B)	1×10^{-5} mol L^{-1}	0.0037
(C)	5×10^{-5} mol L^{-1}	0.0061

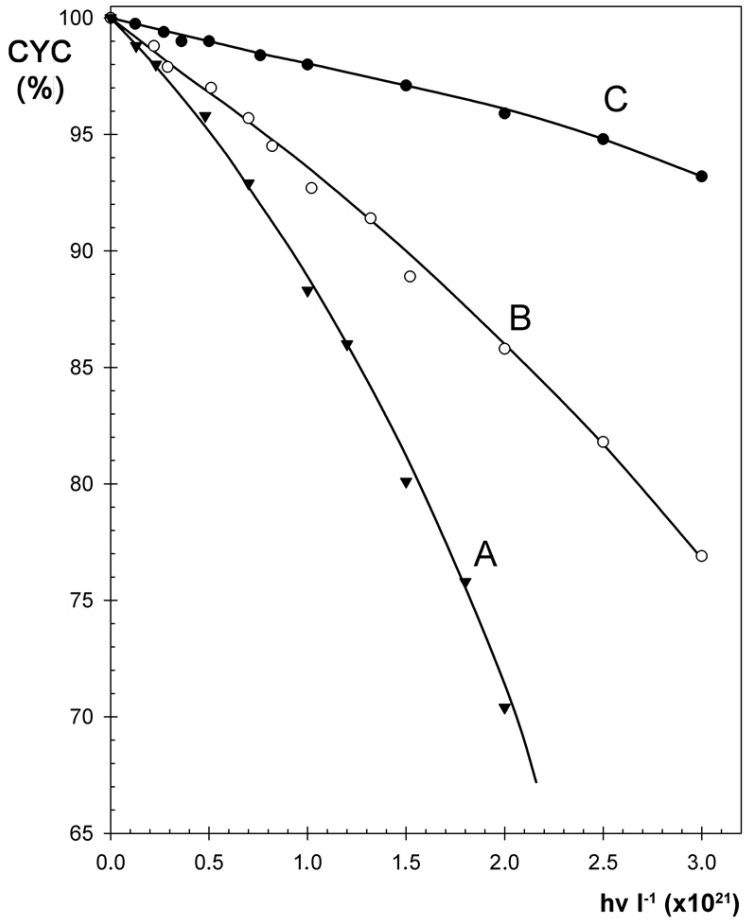
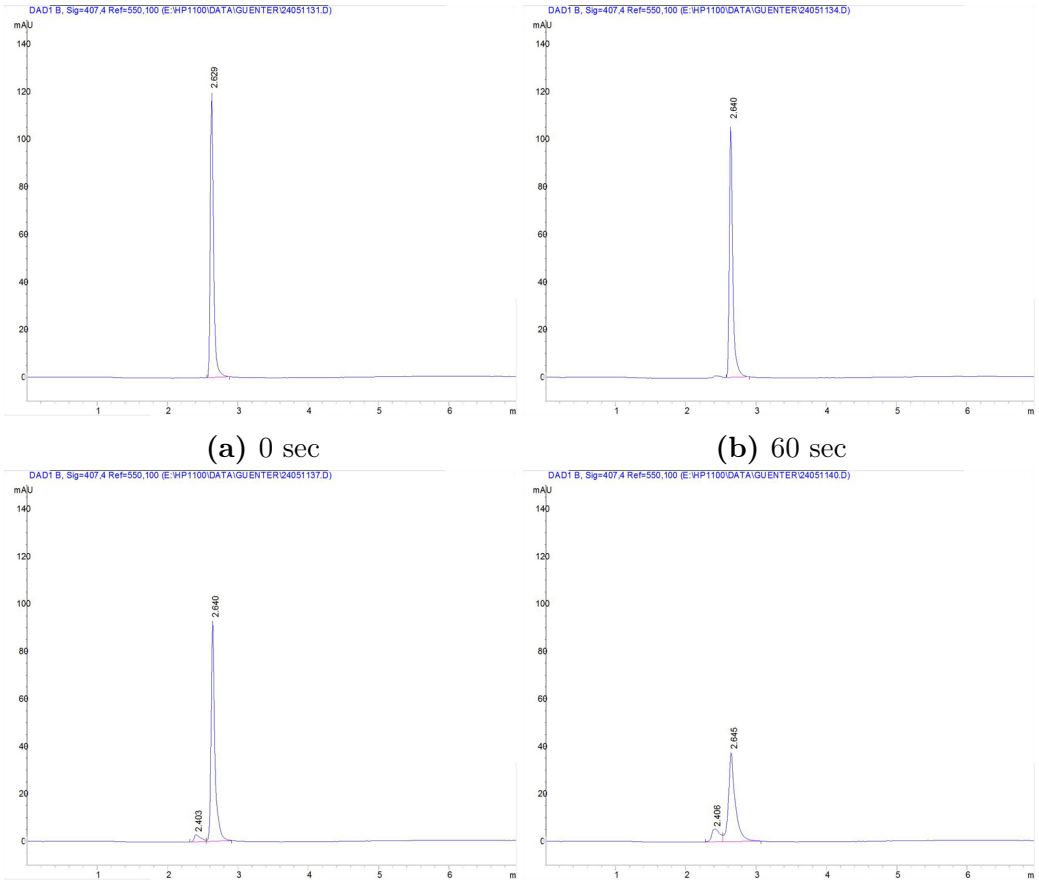


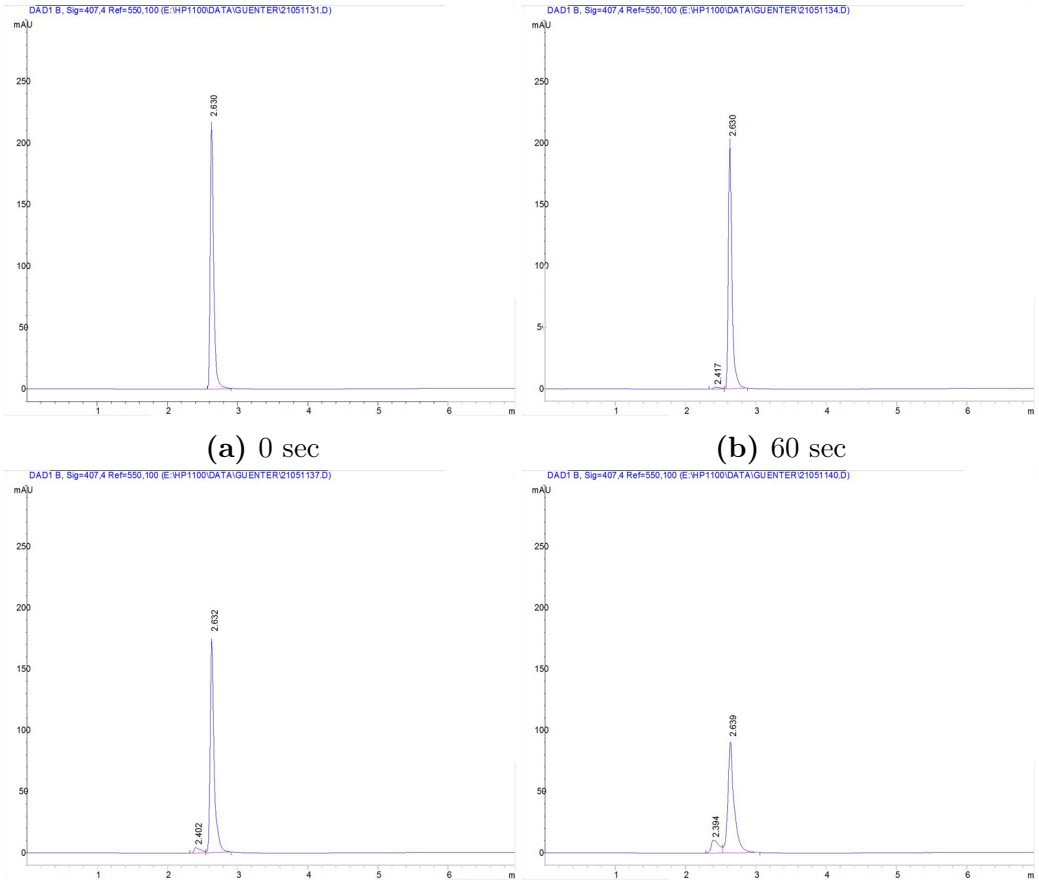
Figure 3.5 – Photolysis of CYC in % in airfree solution, determined by HPLC. Degradation of CYC is presented as a function of absorbed UV dose ($h\nu$ L^{-1}); (A) 5 $\times$ 10^{-6} mol L^{-1}, (B) 1 $\times$ 10^{-5} mol L^{-1} and (C) 5 $\times$ 10^{-5} mol L^{-1}.



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

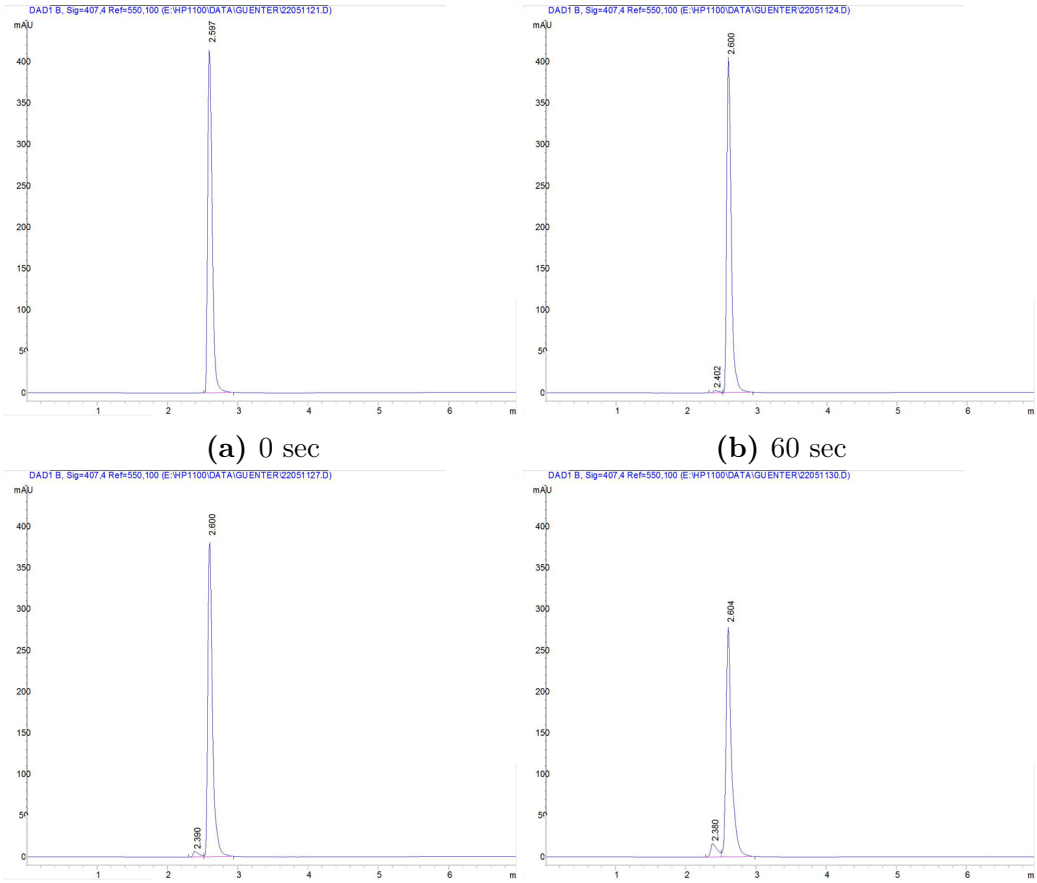
Figure 3.6 – HPLC chromatograms of photolysis of 1×10^{-6} mol L^{-1} CYC in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.7 – HPLC chromatograms of photolysis of 1×10^{-5} mol L^{-1} CYC in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.8 – HPLC chromatograms of photolysis of 5×10^{-5} mol L^{-1} CYC in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.

3.3.1.2 Photolysis of CYC - in aerated media

The photolysis of CYC in aerated solution has also been analyzed. Therefore, concentrations of 5×10^{-6} mol L^{-1} and 1×10^{-5} mol L^{-1} CYC were chosen. The degration curves show a little different behavior compared to CYC in airfree solution. They are flattening earlier and do not show the same decreasing rates as well as metabolite formation is observed earlier. Chromatograms are given in figures 3.10 and 3.11. The calculated quantum yields of photodegradation and product formation are given in tables 3.7 and 3.6.

Table 3.6 – Initial quantum yields of photolysis of CYC irradiated in aerated solution. (A) and (B) are indicating the corresponding curves in figure 3.9.

Curve	CYC	Q_i(CYC)
(A)	5×10^{-6} mol L^{-1}	0.0004
(B)	1×10^{-5} mol L^{-1}	0.0013

Table 3.7 – Initial quantum yields of formation of photolytic products of CYC irradiated in aerated solution. (A1) and (B1) are indicating the corresponding curves in figure 3.9.

Curve	CYC	$Q(CYC)_i$
(A1)	5×10^{-6} mol L^{-1}	0.0005
(B1)	1×10^{-5} mol L^{-1}	0.0018

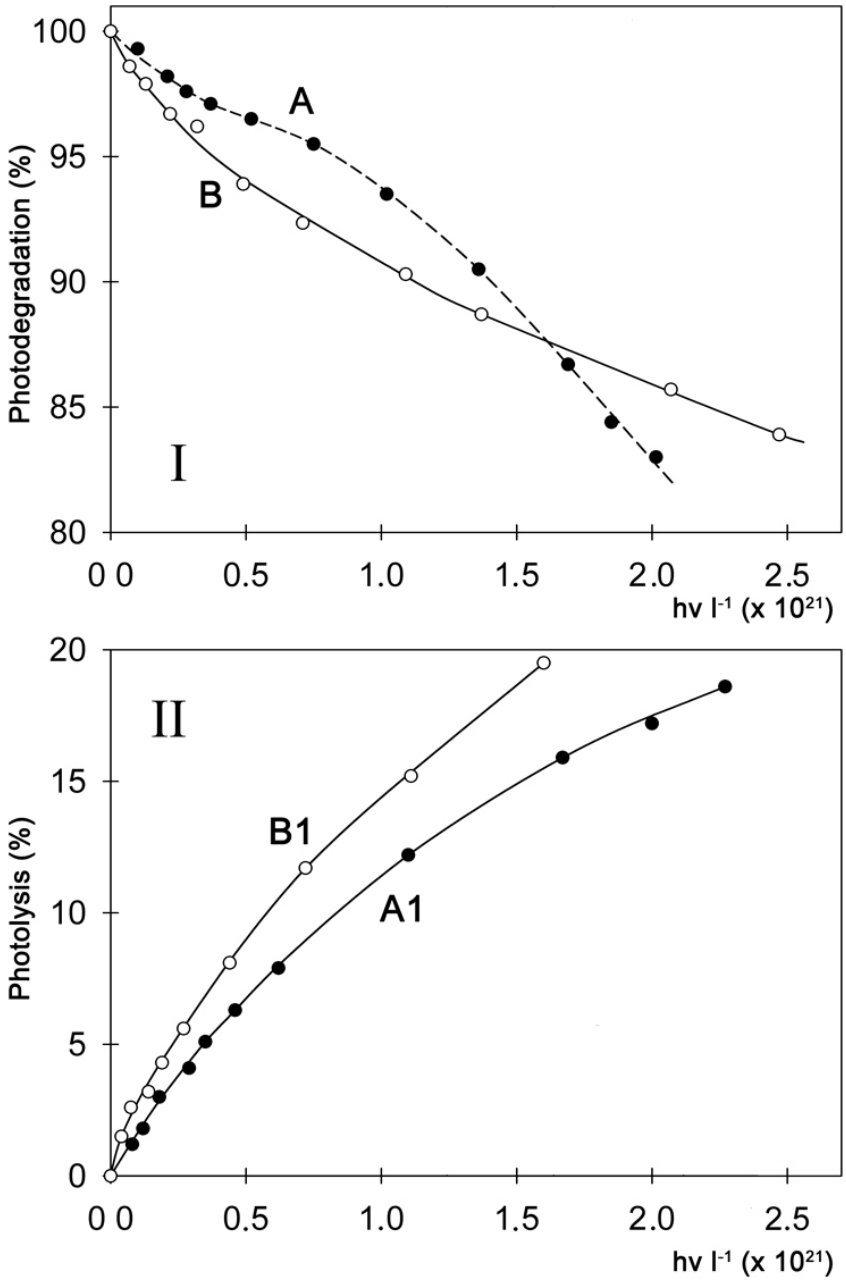
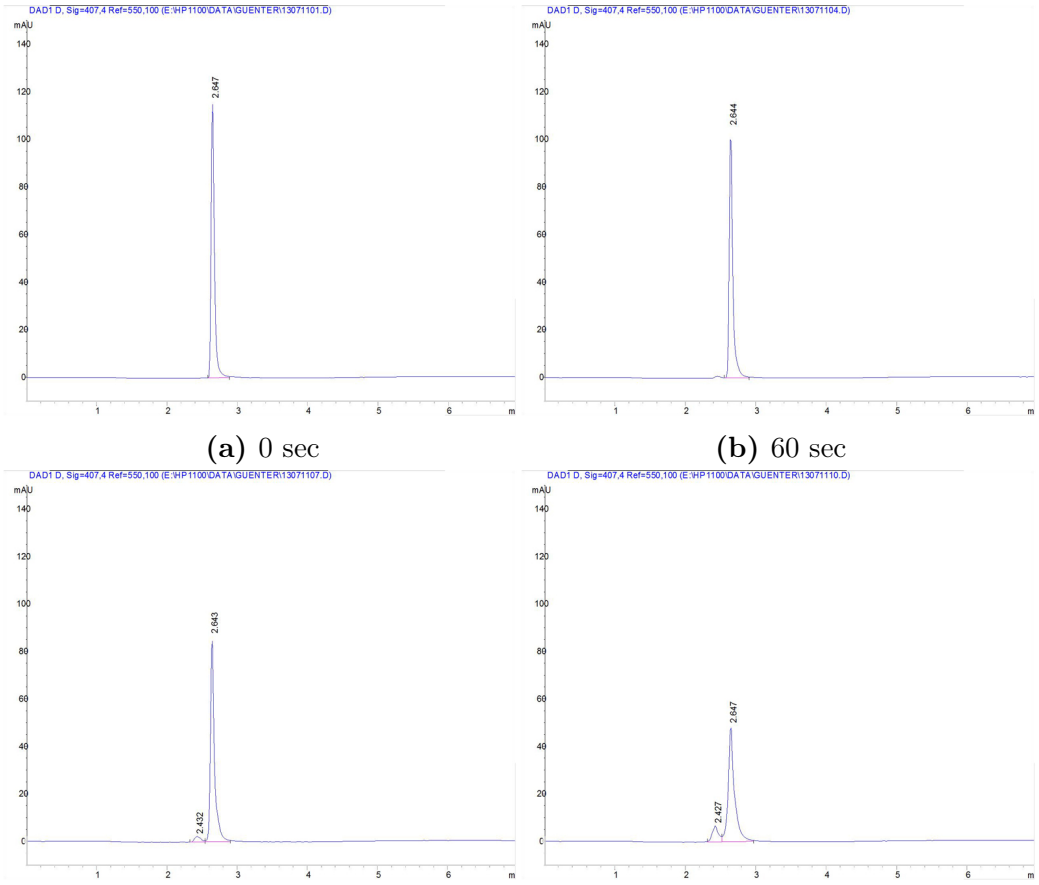


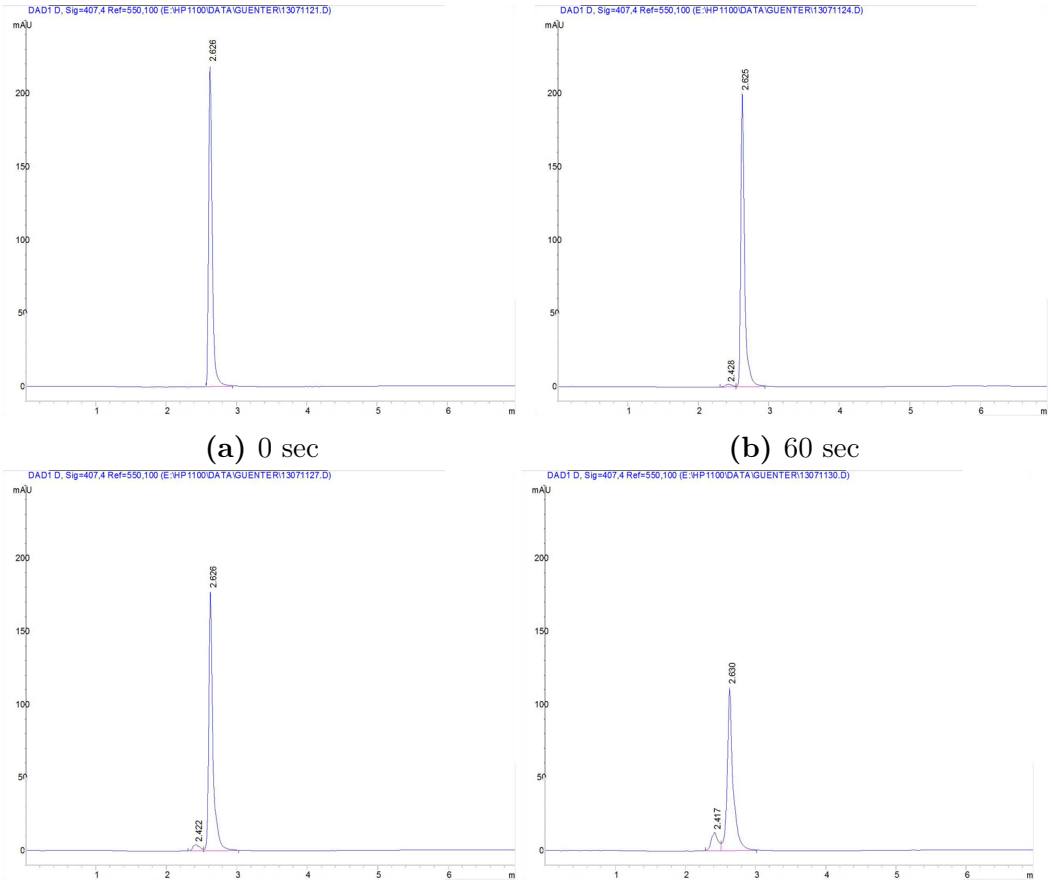
Figure 3.9 – HPLC analysis - photodegradation and product formation of CYC in aerated solution (pH 7.4). [A] & [A1] 5×10^{-6} mol L^{-1} CYC and [B] & [B1] 1×10^{-5} mol L^{-1} CYC. Diagram (I) is showing the degradation of CYC (%) and (II) the formation of photolytic products (%).



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.10 – HPLC chromatograms of photolysis of 5×10^{-6} mol L^{-1} CYC in aqeous aerated solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.11 – HPLC chromatograms of photolysis of 1×10^{-5} mol L^{-1} CYC in aqeous aerated solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.

3.3.2 Ascorbic Acid

VitC is a quite unstable substance in aqueous solutions, degrading without additional impacts. Being treated with UV light it gets degraded faster, emitting e^-_{aq} . At a concentration of 1×10^{-5} mol L^{-1} almost all of VitC is depleted after consumption of 2×10^{21} hν L^{-1}, see figure 3.12. The chromatograms of the HPLC measurements are given in figures 3.13 and 3.14. Concentrations tested were: 1×10^{-5} mol L^{-1}, 5×10^{-5} mol L^{-1} and 1×10^{-4} mol L^{-1} VitC. The retention time of VitC was 0.21 min.

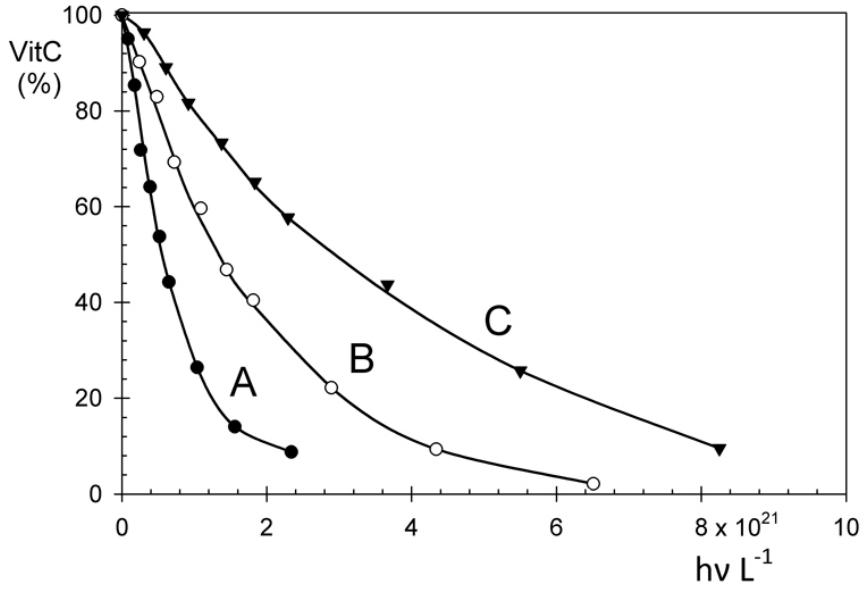
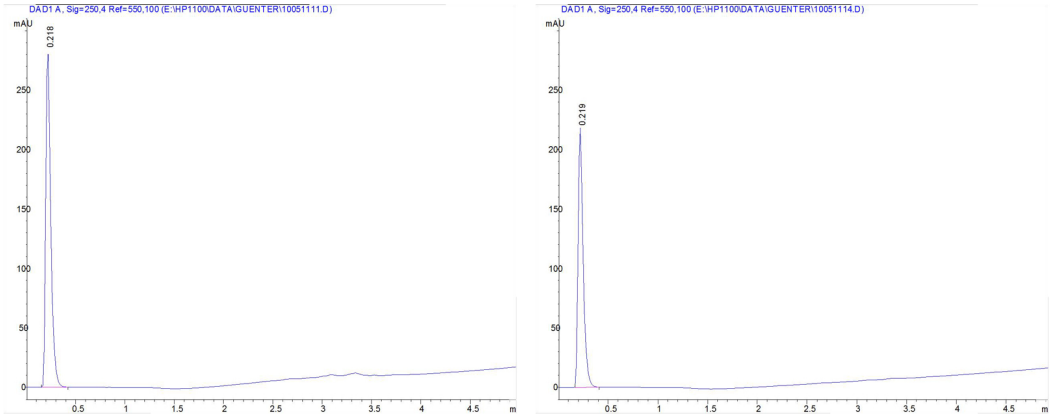


Figure 3.12 – HPLC analysis - photolysis of VitC as a function of absorped UV-dose (hν L^{-1}). (A) 1×10^{-5} mol L^{-1}, (B) 5×10^{-5} mol L^{-1} and (C) 1×10^{-4} mol L^{-1} (pH 7.4).

3.3.3 Cytochrome c and Ascorbic Acid in Mixture

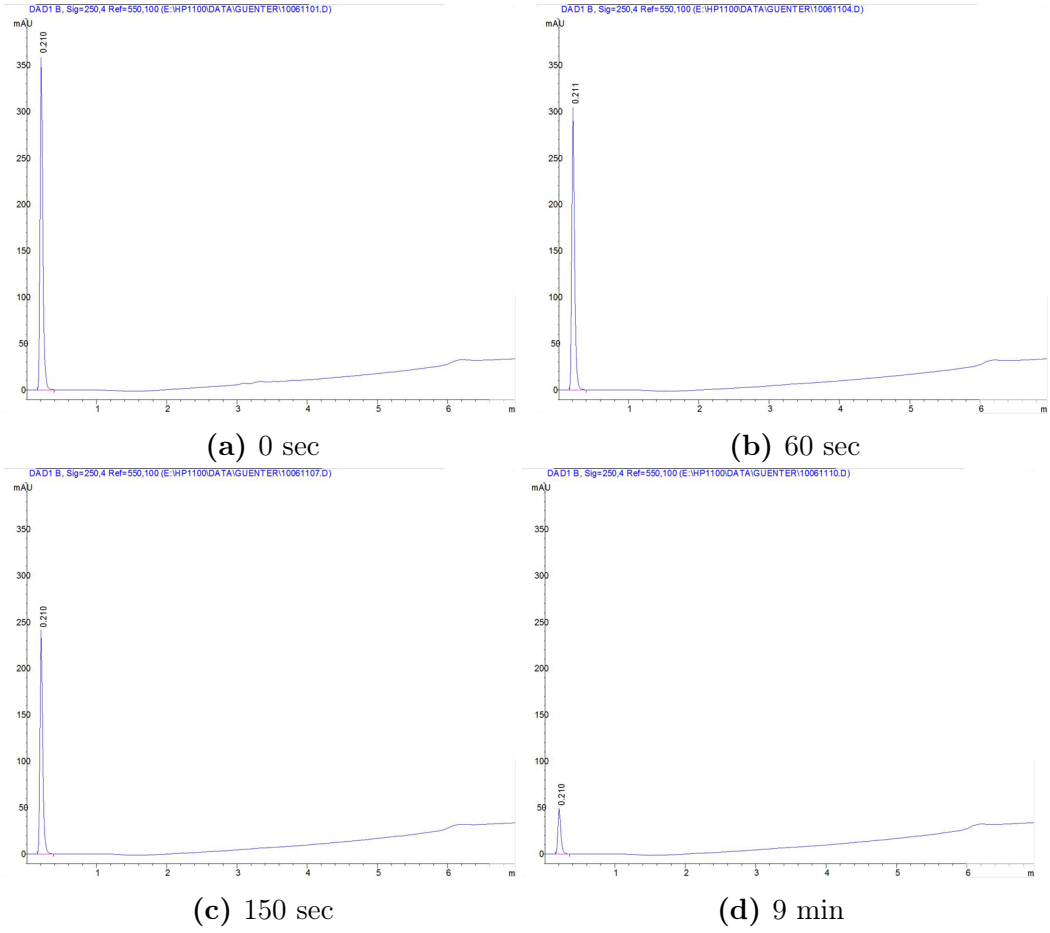
Concerning previous experiments the behaviour of CYC in mixture with VitC was also of interest. Tested concentrations of mixtures are given in figure 3.15 and table 3.8. Figure 3.15 shows the remainder (%) as a function of absorbed UV dose, where curve (A) and (A1) are presenting VitC at 250 nm and (B) and (B1) CYC (corresponding concentrations are given in table 3.8). It seems that CYC and VitC



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.13 – HPLC chromatograms of photolysis of 5×10^{-5} mol L^{-1} VitC in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



(a) 0 sec **(b)** 60 sec

(c) 150 sec **(d)** 9 min

Figure 3.14 – HPLC chromatograms of photolysis of 1×10^{-4} mol L^{-1} VitC in aqeous aerated solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.

in mixture are photolysed way faster than being irradiated in solution solo. The initial quantum yields are given in table 3.8. Metabolite formation occurs quite fast, whereby development of two different products is recognized, see figure 3.16. The primary product (P1) occurs right after beginning of UV-treatment. With ongoing UV treatment it gets degraded as well, while a secondary product (P2) shows up. The increase of the CYC curve (A) might be a result of interactions of CYC with its products. HPLC chromatograms of different mixture ratios at UV-duration of 0, 60 and 150 sec as well as of 9 min are given in figures 3.17, 3.18 and 3.19. On the left of the figures the 250 nm line is shown, representing VitC, on the right CYC is given at 407 nm. The depletion of VitC and the formation of the metabolites through CYC degradation are well represented.

Table 3.8 – Q_i of mixures of CYC + VitC

Curve	CYC	VitC	Q_i
(A)		1×10^{-5} mol L^{-1}	0.0542
(A1)	1×10^{-5} mol L^{-1}	1×10^{-5} mol L^{-1}	0.0265
(B)	1×10^{-5} mol L^{-1}		0.0295
(B1)	1×10^{-5} mol L^{-1}	1×10^{-5} mol L^{-1}	0.0270

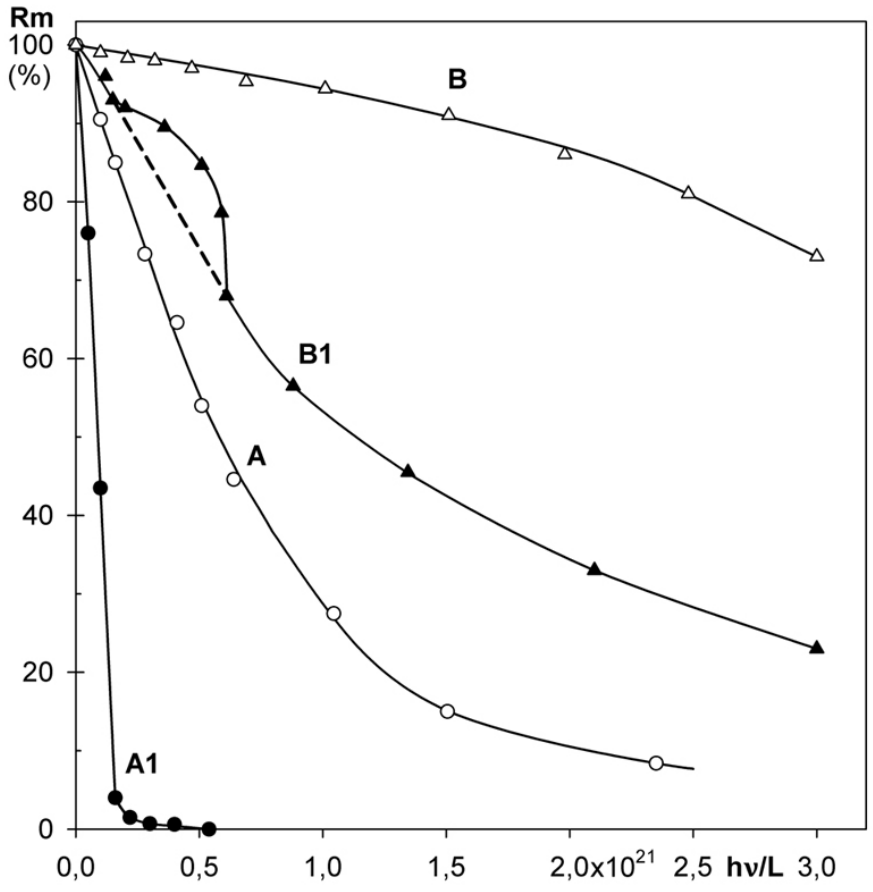


Figure 3.15 – Remainder of photolysis in % (Rm(%)) in airfree aqueous solution (pH 7.4). (A) 1×10^{-5} mol L^{-1} VitC, (A1) 1×10^{-5} mol L^{-1} Vitc in combination with 1×10^{-5} mol L^{-1} CYC, (B) 1×10^{-5} mol L^{-1} CYC and (B1) 1×10^{-5} mol L^{-1} CYC in combination with 1×10^{-5} mol L^{-1} VitC.

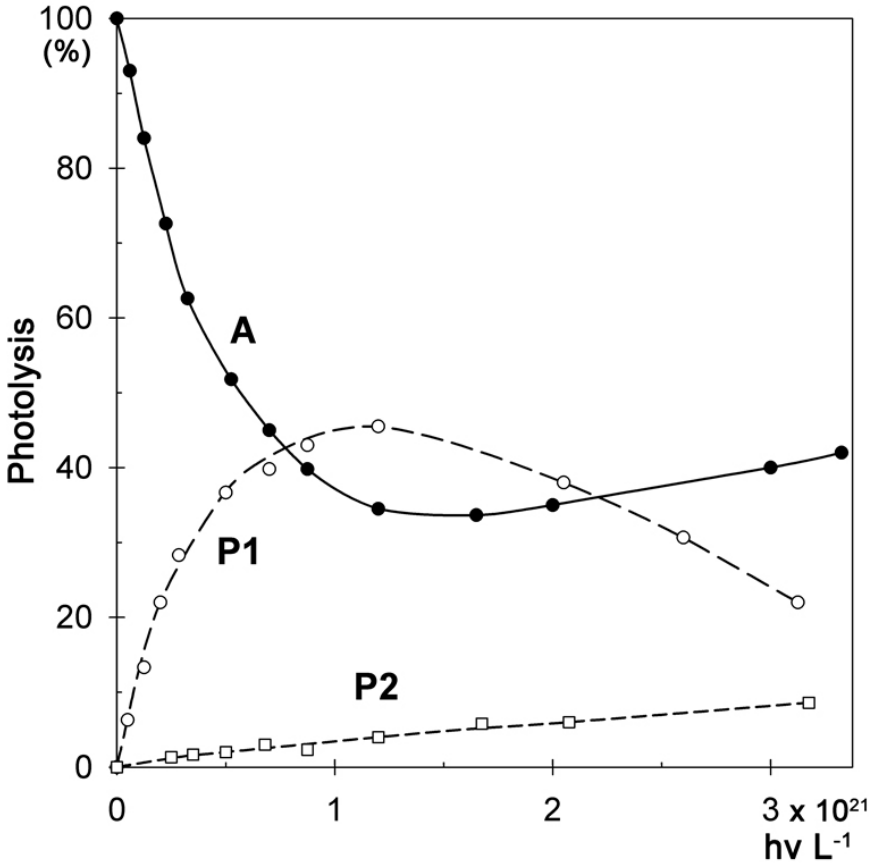
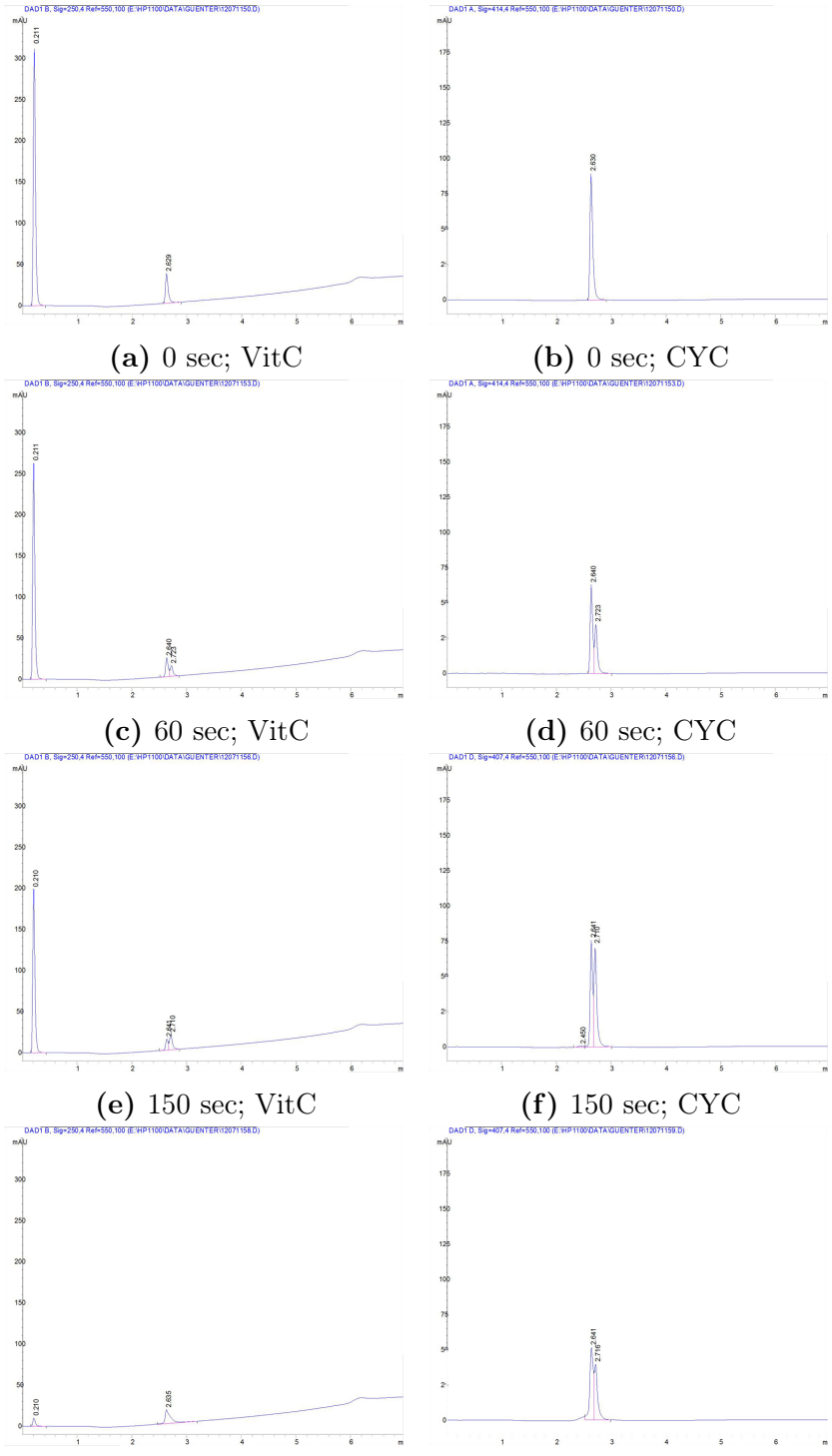


Figure 3.16 – HPLC analysis of photolysis of 1×10^{-5} mol L^{-1} CYC + 5×10^{-5} mol L^{-1} VitC in airfree aqueous solution (pH7.4). (A) photolysis of mixture, (P1) primary product and (P2) secondary product development.



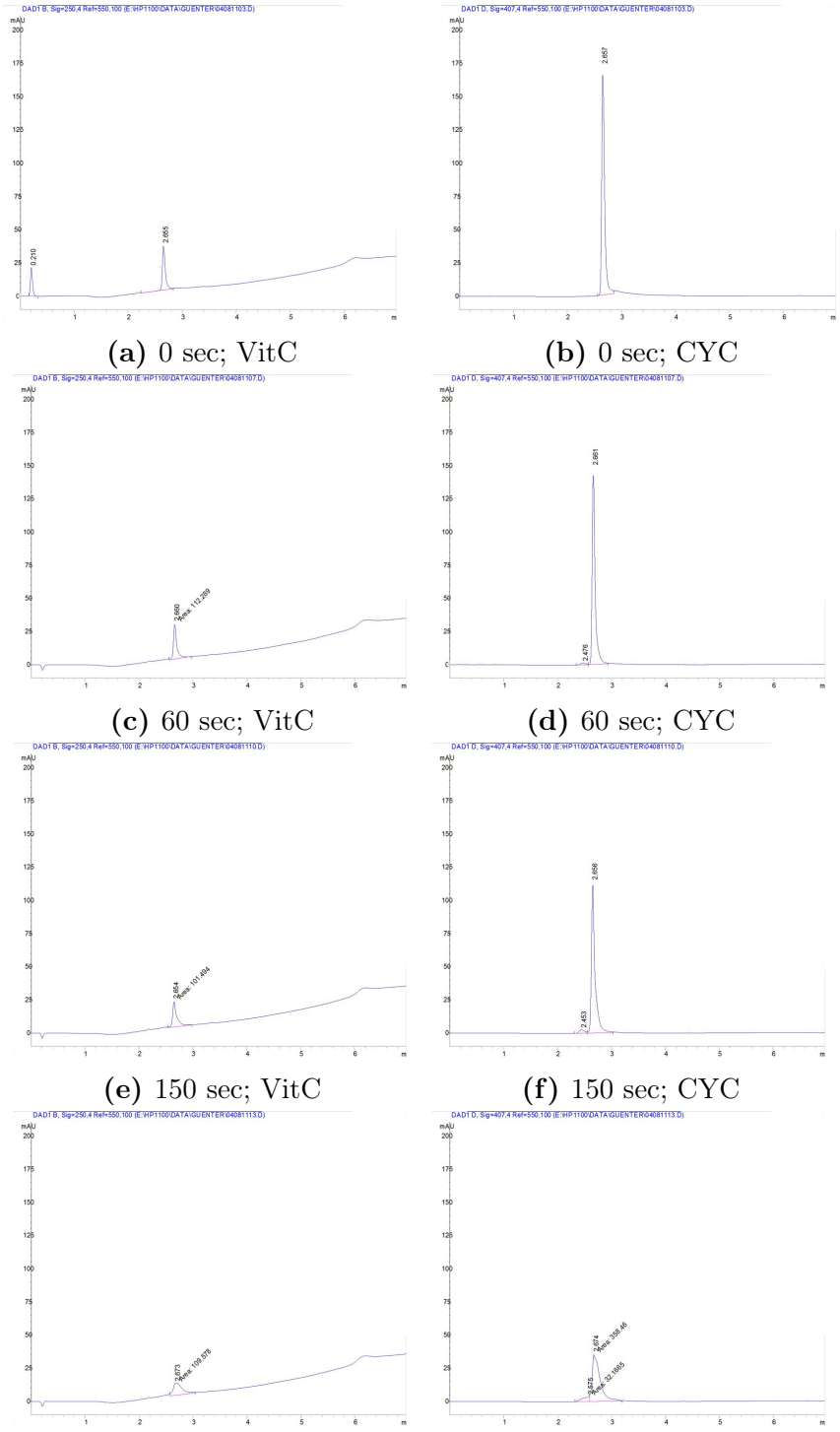
(a) 0 sec; VitC **(b)** 0 sec; CYC

(c) 60 sec; VitC **(d)** 60 sec; CYC

(e) 150 sec; VitC **(f)** 150 sec; CYC

(g) 9 min; VitC **(h)** 9 min; CYC

Figure 3.19 – Photolysis. HPLC chromatograms of a mixture of 1×10^{-4} mol L^{-1} VitC (left) and 1×10^{-5} mol L^{-1} CYC (right) in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



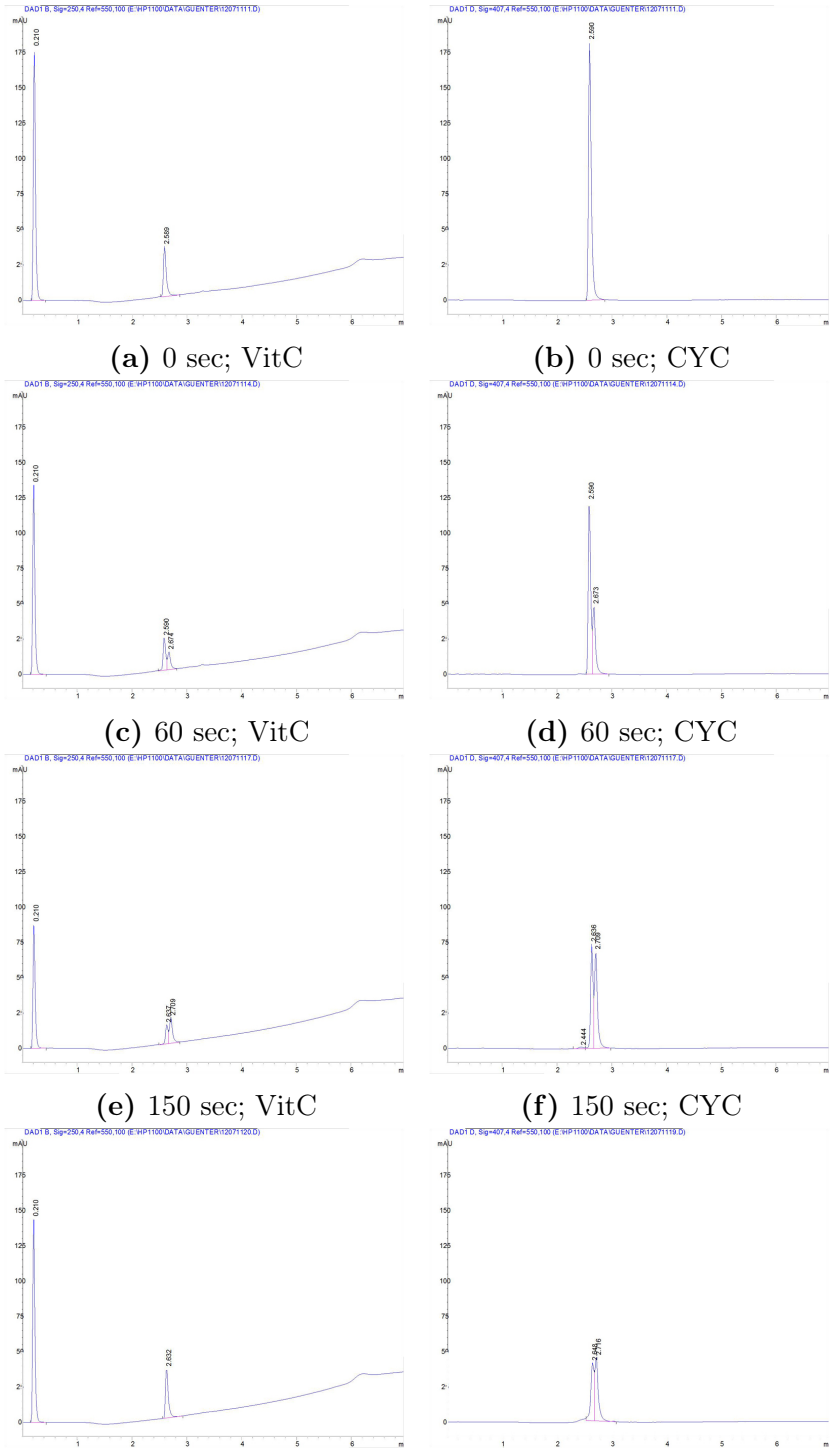
(a) 0 sec; VitC **(b)** 0 sec; CYC

(c) 60 sec; VitC **(d)** 60 sec; CYC

(e) 150 sec; VitC **(f)** 150 sec; CYC

(g) 9 min; VitC **(h)** 9 min; CYC

Figure 3.17 – HPLC chromatograms of a mixture of 1×10^{-5} mol L^{-1} VitC (left) and 1×10^{-5} mol L^{-1} CYC (right) in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.



(a) 0 sec; VitC **(b)** 0 sec; CYC

(c) 60 sec; VitC **(d)** 60 sec; CYC

(e) 150 sec; VitC **(f)** 150 sec; CYC

(g) 9 min; VitC **(h)** 9 min; CYC

Figure 3.18 – HPLC chromatograms of a mixture of 5×10^{-5} mol L^{-1} VitC (left) and 5×10^{-5} mol L^{-1} CYC (right) in aqeous airfree solution. Timepoints given: 0, 60 and 150 sec as well as 9 min.

3.4 In Vitro Experiments

In vitro experiments were performed with MCF-7 breast cancer cells. They were treated with CYC, VitC and a mixture of both and subsequently irradiated with γ-rays at doses of 0, 5, 10, 15, 20, 25 and 30 Gray.

3.4.1 Toxicity Test

The toxicity tests performed with the different concentrations chosen did not show any effect on the growth of the cell culture with the exception of VitC supplement in the high dose of 5×10^{-4} mol L^{-1}. Cultures treated with that concentration showed a slower growth and their appearance was slightly affected. At a $c_{VitC} = 1 \times 10^{-4}$ mol L^{-1} there was no difference in the appearance of the cells but a little slower growth rate compared to cultures with lower c_{VitC} was recognized. This might be mainly due to the acidification of the culture medium by VitC ($pK_s = 4.25$), where at higher concentrations of VitC the buffering of the culture medium is not sufficient enough.

Based on the test results following concentrations where chosen for the in vitro experiments:

Table 3.9 – Concentrations chosen for in vitro experiments on MCF-7 cells after toxicity tests.

solo:	**CYC mol/L**	**VitC mol/L**	**mix:**	**CYC mol/L**	+	**VitC mol/L**
	5×10^{-6}	5×10^{-6}		5×10^{-6}	+	5×10^{-6}
	1×10^{-5}	5×10^{-5}		5×10^{-5}	+	1×10^{-5}
		1×10^{-4}		1×10^{-5}	+	1×10^{-6}

3.4.2 Substance Treatment

Substances were tested in concentrations as shown in table 3.9. The survival rates are given as ratio

$$\frac{N}{N_0} \tag{3.3}$$

where
N is the survival rate of the irradiated cells and
N_0 is the cell number of the unirradiated control group.

MCF-7 cells were treated with CYC and VitC about one hour before irradiation. The applied doses were: 0, 5, 10, 15, 20, 25 and 30 Gray.

3.4.3 Control group

The control group showed following survival rates after irradiation:

Table 3.10 – Survival ratios of MCF-7 cells without substance treatment after γ-irradiation.

Gray	N/N_0
0	1.000
5	0.344
10	0.200
15	0.157
20	0.136
25	0.132
30	0.119

As expected, most of the cells are already killed at a dose of 5 Gy (3.20). The D_{37} value is achieved by a dose of 4.6 Gy (see figure 3.21). Figure 3.20 is representing cell cultures after γ-ray treatment at doses of 0, 10, 20, and 30 Gray. At a dose of 10 Gy most of the cells got killed and at a dose of 30 Gy almost none are left alive. Figure 3.20 is representative for all following in vitro experiments as no obvious visual difference is noticeable between the differently treated cultures.

3.4.4 Cytochrome c

While CYC in a lower concentration, in comparison to the control group, does not seem to be protective, a higher amount of CYC in the culture medium has a slight effect on the survival rate of MCF-7 cells. Compared to the D_{37} of the untreated control group (4.6 Gy), the D_{37} of cells treated with 1×10^{-5} is higher,

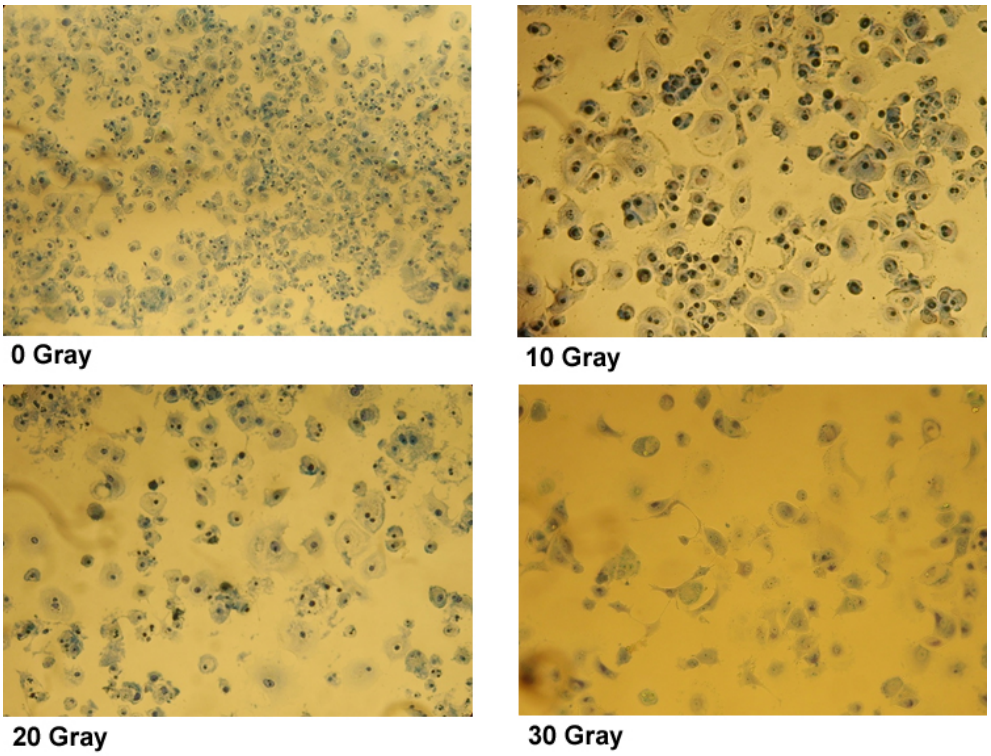


Figure 3.20 – Untreated cells after irradiation with γ-rays. Pictures show cell cultures after treatment with 0, 10, 20 and 30 Gray, at a 100 fold magnification. The control group (0 Gray) is showing a dense population with small cells. Already at 10 Gray most of the cells are killed and at 30 Gray almost no cells are left alive. Large inflatet cells are a typical appeareance after irradiation indicating a soon cell death.

D_{37} = 5 Gy (figure 3.21). At higher irradiation doses the survival rate decreases below the survival rate of the control group. The survival rates after γ-irradiation, determined for the different concentrations of CYC, are given in table 3.11.

3.4.5 Ascorbic Acid

As ascorbic acid is known as irradiation protective agent, due to its function as radical scavenger, it is of no surprise that the survival rate is increasing compared to the control group. Higher concentrations of VitC have an effect on the D_{37}, which is 4.9 Gy at a concentration of 1×10^{-4} (figure 3.21).

Table 3.11 – Survival ratios of MCF-7 cells after CYC treatment and γ-irradiation.

Gray	N/N_0 5×10^{-6}	N/N_0 1×10^{-5}
0	1.000	1.000
5	0.344	0.370
10	0.179	0.201
15	0.142	0.145
20	0.138	0.129
25	0.126	0.123
30	0.120	0.108

The treatment with VitC leads to following survival numbers after irradiation:

Table 3.12 – Survival ratios of MCF-7 cells after VitC treatment and γ-irradiation.

Gray	N/N_0 5×10^{-6}	N/N_0 5×10^{-5}	N/N_0 1×10^{-4}
0	1.000	1.000	1.000
5	0.328	0.362	0.377
10	0.226	0.220	0.221
15	0.192	0.180	0.186
20	0.181	0.150	0.177
25	0.159	0.137	0.153
30	0.136	0.122	0.150

3.4.6 Cytochrome c and Ascorbic Acid in Mixture

Cell treatment with a mixture of both substances, CYC and VitC, shows lower survival rates compared to the control group and the groups just treated with one of the substances. It seems that CYC in combination with VitC is developing products, increasing the effect of γ-ray irradiation. When treated with a mixture of CYC and VitC MCF-7 cells showed after γ-irradiation the survival rates given in table 3.13.

The ratios of the most effective substrate concentrations are given as survival curves in figure 3.21. The D_{37} values of the differently treated MCF-7 cells are displayed enlarged in the inset.

Table 3.13 – Survival ratios of MCF-7 cells after treatment with a mix of CYC and VitC and γ-irradiation.

Gray	N/N_0 5×10^{-6} CYC 5×10^{-6} VitC	N/N_0 1×10^{-5} CYC 1×10^{-5} VitC	N/N_0 1×10^{-5} CYC 5×10^{-6} VitC
0	1.000	1.000	1.000
5	0.283	0.294	0.329
10	0.276	0.207	0.215
15	0.218	0.135	0.163
20	0.151	0.121	0.146
25	0.126	0.121	0.135
30	0.112	0.120	0.121

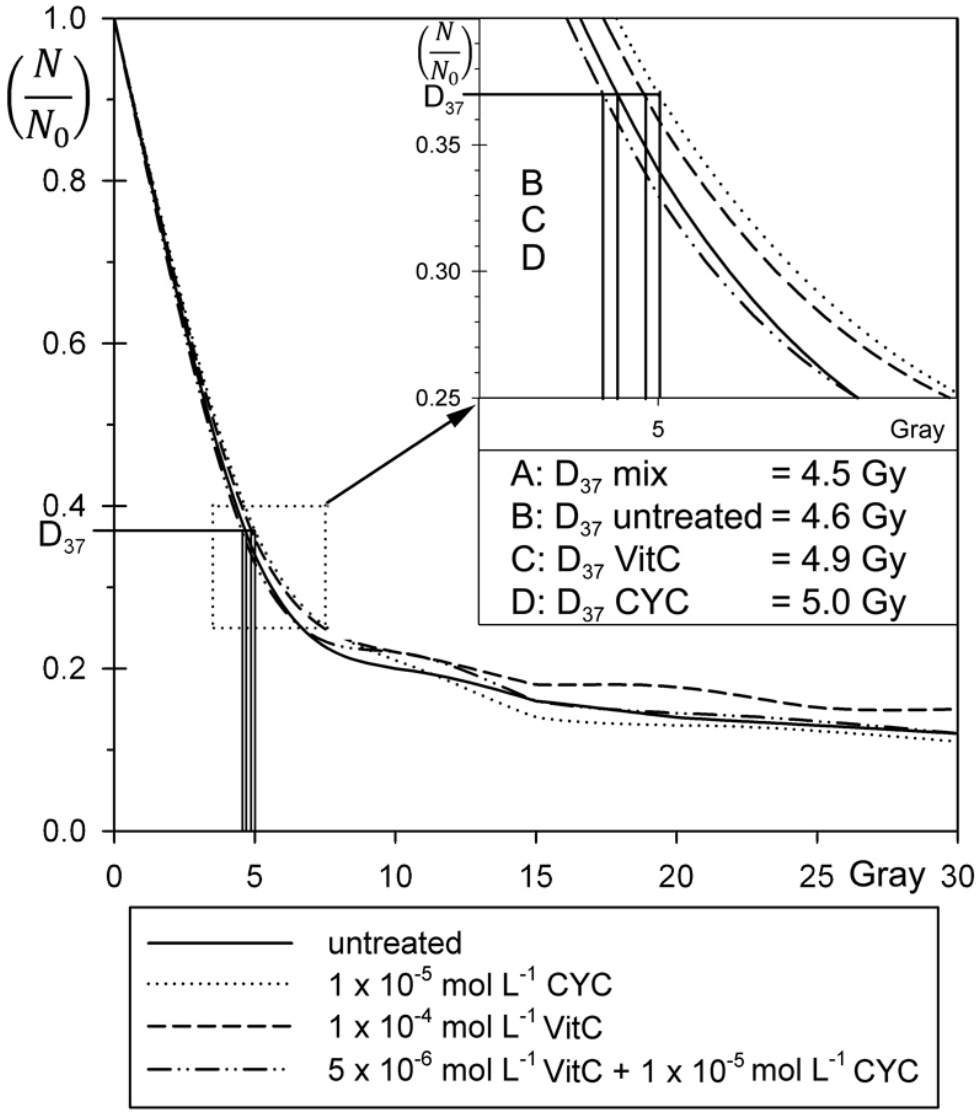


Figure 3.21 – Survival curves after substance treatment and irradiation. Survival curves of cells treated with 1×10^{-5} mol L^{-1} CYC, 5×10^{-6} mol L^{-1} VitC and a mix of 5×10^{-6} mol L^{-1} VitC and 1×10^{-5} mol L^{-1} CYC as well as untreated cells are shown. The inset gives an enlarged area for a better view on the D_{37} values. A: D_{37} of the mix of CYC and VitC, which has the lowest value, D_{37} = 4.5 Gray; B: D_{37} of the untreated control group, D_{37} = 4.6 Gray; C: D_{37} of VitC, D_{37} = 4.9 Gray; D: D_{37} of CYC, D_{37} = 5 Gy. According to this data CYC and VitC alone in solution are providing protection against irradiation, while the mix of both seems to be sensitizing, compared to the untreated control group. At higher doses VitC seems to be the most protective reagent. CYC turns to the opposite, resulting in lower cell numbers than the control group, at doses higher than 10 Gy. The mix stays in line to the control group neither with a positve nor a negative effect on survival rates.

Chapter 4

Discussion

4.1 e^-_{aq} Emission from CYC

It was established that CYC, when excited into singlet state in aqueous media, can eject "solvated electrons" (e^-_{aq}). The yield of e^-_{aq} depends on several factors: substrate concentration, temperature etc. At higher CYC concentrations the formation of instable associates is observed, which consume a part of the ejected e^-_{aq} . As a result of the e^-_{aq} emission free radicals are formed, which lead to metabolite formation (3.3). The observed effect is shown in figure 3.4. The two curves do not show a direct proportion to their concentrations, as at higher CYC levels the e^-_{aq} yield is lower then measured at lower concentration. Having a look at the reaction rate constant for reactions of CYC with e^-_{aq} (k =4.5 $\times$ 10^{10} L mol^{-1} s^{-1}) a large part of emitted e^-_{aq} are consumed by CYC itself, resulting in lower Q(e^-_{aq}) yields by increasing CYC concentrations.

The exited e^-_{aq} originate partly from the Fe^{2+} core of the heme, but might also be a result of e^-_{aq} emission of double bonds and the $-$COOH groups (Figure 1.2c). Developed products are additionally contributing to e^-_{aq} but not at the same level as CYC.

4.2 Mutual interaction of CYC with VitC

VitC is also emitting e^-_{aq} . It was used as a representative of biological compounds in the organism, acting as e^-_{aq} donor.

HPLC analyses are showing the degradation of CYC and VitC very well. VitC was observed at $\lambda = 250$ nm and calculations for CYC derived from observation of the $\lambda = 407$ nm line. At lower concentrations the substances are degraded much faster than at higher substance yields. While VitC seems to be degraded completely (Figure 3.12), CYC is forming metabolites as mentioned above (Figure 3.8). Interestingly, the calculated yields of e^-_{aq} ($Q(e^-_{aq})_i$), is for several concentrations much higher compared to the yield of CYC photolysis (Q_{CYC}), see table 4.1.

Table 4.1 – Comparison of $Q(e^-_{aq})_i$ and Q_{CYC}.

mol L^{-1}	$Q(e^-_{aq})_i$	Q_{CYC}
5×10^{-6}	0.0186	0.0003
1×10^{-5}	0.0115	0.0037
5×10^{-5}	0.0072	0.0061

An explanation could be given by the fact that e^-_{aq} are not only deriving from the Fe-core, but also from excited double bonds or $-$COOH groups of the porphyrin. Thereby CYC radical is formed ($CYC^{\bullet+}$). This species is very reactive and can react with water leading to regeneration of CYC, see equations 4.1 and 4.2. The thereby produced $OH^\bullet$ are then scavenged by the added chloroethanol (4.3) or partly by CYC itself (equations 4.4 and 4.5) in the irradiation solution.

$$CYC \quad \rightsquigarrow (CYC)^* \quad \rightarrow e^-_{aq} + CYC^{\bullet+} \tag{4.1}$$

$$CYC^{\bullet+} + H_2O \quad \rightarrow CYC + H^+ + OH^\bullet \tag{4.2}$$

$$OH^\bullet \quad + ClC_2H_4OH \rightarrow ClC_2H_4OH^\bullet + H_2O, (k = 9.5 \times 10^8\ L\ mol^{-1}\ s^{-1}[4]) \tag{4.3}$$

$$OH^\bullet \quad + CYC \quad \rightarrow CYC - OH(OH - adduct) \tag{4.4}$$

$$\rightarrow CYC^\bullet + H_2O, (k = 1.4 \times 10^{10}\ L\ mol^{-1}\ s^{-1}[4]) \tag{4.5}$$

Kinetic calculations showed a reaction of 91 % of $OH^\bullet$ with chloroethanol,

while only 9 % are reacting with CYC, explaining the differences in the quantum yields. By this considerations the low Q_{CYC} is explainable.

Table 4.2 shows the determined percentage of scavenged e^-_{aq} in the different mixtures tested for CYC and chloroethanol. Chloroehtanol is obviously scavenging most of the emitted e^-_{aq} while CYC just consumes a small part of it, but at higher concentrations of CYC, its fraction of e^-_{aq} consumption is increasing.

Table 4.2 – Kinetic calculations.

Mix	mixture (mol L^{-1})	scavenged e^-_{aq} %
A	1×10^{-2} ClC_2H_4OH	97.0
	5×10^{-6} CYC	3.0
B	1×10^{-2} ClC_2H_4OH	93.4
	1×10^{-5}	6.6
hline C	1×10^{-2} ClC_2H_4OH	74.0
	5×10^{-5}	26.0

CYC is also known as an antioxidant, which was proven by this work. It was interesting to compare CYCs ability with the one of VitC, which is known as very strong antioxidant substance. Therefore, the remainder (%) of VitC and CYC solo in solution as well as of a mixture of both of them after photolysis where analysed by HPLC. Figure 3.15 is presenting the results of this analysis. Curve (A) is presenting 1×10^{-5} mol L^{-1} VitC and (B) the same concentration of CYC being UV-irradiated solo in airfree aqueous solution. Curves (A1) and (B1) are giving the photodegration of VitC (A1) and CYC (B1) when being treated in a mixture of both at the same concentrations. The Q_i yields of VitC treated in mixture is lower (51 %) than the Q_i of the mixture. VitC is very fast degraded in the mixture, since it acts as a better e^--donor. A e^-_{aq} transfer from VitC to CYC-transients is obviously leading to regeneration of CYC. Having a closer look at the behavior of CYC, solo and in mix with VitC, their $Q(e^-_{aq})$ is also higher then the $Q(e^-_{aq})_i$ of the substrates treated individually (curve B). Calculating the difference indicates that about 8.5 % of the emitted e^-_{aq} of CYC are transferred to VitC. This is showing that a mutual e^-_{aq} transfer is occuring in the mixture of

CYC and VitC.

The formation of products after UV-irradiation of the mixture of CYC and VitC was also analysed by HPLC. VitC is degraded very fast within the mixture, while it looks like CYCs product formation is accelerated (Figures 3.19 and 3.15). The primary product is formed at higher yields right at the beginning of UV irradiation, but degraded by increasing irradiation at a dose of 1.2×10^{21} hν L^{-1}, while secondary product is steadily formed at low yields. Additionally, it seems that CYC interacts with some of the metabolites, as its yield is slightly increasing after an UV dose of 1.5×10^{21} hν L^{-1} (Figure 3.16).

The discussed results are published in the paper "Cytochrome c: Electron Emission, Photodegradation and Mutual Interaction with Vitamin C", by N. Getoff and G. Walder at the journal In Vivo [63].

4.3 Experiments In Vitro

A higher survival rate at D_{37} is observed of irradiated samples treated with CYC, in contrast to the untreated control group. Figure 3.21 shows that there is a slightly protective effect of CYC, with a D_{37} of 5 Gy compared to the control group with a D_{37} of 4.6 Gy. Addition of only VitC has a positive impact on the D_{37} (4.9 Gy), but the expected boosting of the survival rate of the mix of both substances did not occur. Treatment with the mixture of both on the contrary led to a lower D_{37} (4.5 Gy) compared to the control group. This may be a consequence of VitC being consumed by CYC, loosing its protective effect, while CYC seems to be degraded somehow faster leading to a decrease of its protective activity or the development of toxic metabolites. The metabolite formation was shown via HPLC analysis, see chapter 4.2. This happens faster in the combination with VitC than CYC being irradiated alone.

Another interesting fact is given by the survival ratios at higher irradiation doses, having a closer look on the numbers of tables 3.11, 3.12 and 3.13 and at the survival curves in figure 3.21. They show that CYC can be protectiv at low irradiation doses, but more destructive at higher doses, compared to the control group. The change from protective to destructiv occurs at an irradiation dose of about 10 Gy. This may derive from the development of metabolites, which might

enhance the impact of irradiation and contribute to the effect experienced at the mixture of CYC and VitC. The development of metabolites was shown by HPLC analysis (3.16). VitC instead shows a constant protective activity with higher survival rates throughout all doses tested.

4.4 Conclusion

As established CYC is able to emit e^-_{aq} being excited in singlet state by irradiation with monochromatic UV-light (λ = 254 nm; E = 4.85 eV/hν; 1 eV = 23 kcal/mol). The e^-_{aq} emission results in CYC-free-radicals, which leads to degradation of the molecule. CYC can consume a part of emitted e^-_{aq} with a rate constant of: k(e^-_{aq} + CYC) = 1.0 x 10^{10} L mol^{-1} s^{-1}, pH 7) and in addition to this, certain parts of e^-_{aq} can be transferred to other biological systems present in the media as well. The presence of antioxidant vitamins, e.g. VitC, acting as e^-_{aq} donor, is leading to degradation of CYC, which is obviously caused by a surplus of e^-_{aq} , attacking CYC.

Experiments in vitro with MCF-7 cells in the presence of CYC and VitC applied individually, demonstrate a protective effect under the influence of γ-rays. However, using a mixture of CYC and VitC is leading to a complete diminishment of the protective effect. The thereby resulting radiation products of CYC have a negative impact on the survival of irradiated MCF-7 cells. The CYC products resulting at higher radiation doses are leading to a stronger deactivation of the MCF-7 cells, compared to the control group, especially when a mix of CYC and VitC is applied.

Summary

Cytochromes are playing a vital role within metabolic reactions in the organism. There are a lot of different functions corresponding to the specific action of various cytochromes. In the frame of the research project, cytochrome P450 (CYP) was of special interest, because of its role in hormone transport and metabolism. CYP is, for example, a key compound in the synthesis of dopamin as well as in the pathway of hydroxlation and activation of progesterone. As an appropriate model for cytochromes, cytochrome c (CYC) was used throughout the experiments. Cytochromes are often operating within redox systems being reduced or oxidized by donating or consuming as well as transferring of e^-_{aq} to other biological systems. Thereby ascorbic acid (VitC) was used as a potent electron donor in respect to study its influence on CYC activity. VitC is a very strong antioxidant, and known for its important role of scavenging free radicals within the cell plasma, protecting membrane lipids and other important molecules within the cell from oxidation.

In order to simulate the biological processes in the frame of the studies, monochromatic UV-light at λ = 253.7 nm (E = 4.85 eV/hν; 1 eV = 23 kcal mol^{-1}) was used to excite the molecules into singlet state in order to initiate the above mentioned processes. Experiments in vitro were performed using MCF-7 breast cancer cells under the influence of γ-irradiation to get a deeper insight of the processes involved in the cells.

It was shown that CYC is able to emit e^-_{aq} when excited into singlet state. Thereby, CYC-free radicals are resulting, which are leading to metabolite formation. The application of VitC individually is leading to a protective effect on MCF-7 cells . The same effect was observed by using CYC separately in the presence of the cells. However, in mixture of both substances (CYC and VitC) a strong deactivation of the cells is resulting.

It might be mentioned that in addition to the above mentioned results observed under γ-treatment, also the formation of primary products of water radiolysis (e^-_{aq} , H-atoms, $OH^\bullet$, H_2O_2) is involved in above mentioned processes.

Zusammenfassung

Cytochrome spielen bei vielen Stoffwechselvorgängen im Organismus eine essentielle Rolle. Entsprechend den für jedes Cytochrom spezifischen Reaktionen gibt es unterschiedlichste Aufgaben welche Cytochrome erledigen. Cytochrom P450 (CYP) war im Rahmen dieses Projektes, aufgrund seiner Rollen beim Hormon-Transport sowie -Metabolismus, von großem Interesse. CYP ist zum Beispiel ein zentraler Bestandteil bei der Dopamin Synthese sowie des Hydroxylierungs Verlaufes und der Aktivierung von Progesteron. Als geeignetes Model für Cytochrome wurde Cytochrom c (CYC) während des gesamten Versuchverlaufs verwendet. Cytochrome wirken sehr oft in Redox-Systemen, innerhalb welcher ständige Reduktion und Oxidation durch Abgabe beziehungsweise Aufnahme von e^-_{aq} , aber auch Transfer von e^-_{aq} zu anderen biologischen Systemen stattfindet. Ascorbinsäure (VitC) wurde als potenter Elektronen-Donor zur Untersuchung des Einflusses auf die Aktivität von CYC ausgewählt. VitC is für seine gute antioxidative Wirkung bekannt, und übernimmt eine wichtige Rolle beim Abfangen von gebildeten freien Radikalen innerhalb der Zellen. Auf diese Weise schützt es Membran-Lipide und viele andere essentielle Moleküle innerhalb des Zellplasmas vor Oxidation.

Um im Rahmen der Versuche biologische Prozesse zu simulieren, wurde monochromatisches UV-Licht mit einer Wellenlänge von $\lambda = 253.7$ nm (E = 4.85 eV/hν; 1 eV = 23 kcal mol^{-1}) verwendet um die Moleküle im Singlet-State anzuregen und oben genannte Prozesse zu initiieren. Experimente in vitro wurden mit MCF-7 Brustkrebszellen unter Bestrahlung mit γ-Strahlung durchgeführt, um einen tieferen Einblick in die Abläufe innerhalb der Zellen zu bekommen. Es wurde gezeigt, dass CYC e^-_{aq} emittieren kann wenn es in den Singlet-State angeregt wird. Dabei entwickeln sich freie CYC-Radikale, welche weiters zur Entstehung von Metaboliten führen. Zugabe von VitC bewirkt einen Schutzeffekt auf MCF-7 Zellen. Der gleiche Effekt wurde bei der Verwendung von CYC beobachtet. Sobald aber beide Substancen (VitC und CYC) gemeinsam als Mischung angewandt wurden kam es zu einer starken Deaktivierung der Zellen.

Zusätzlich zu den oben genannten und unter γ-Bestrahlung beobachteten Ergebnissen, ist auch die Bildung von primären Produkten der Wasser Radiolyse (e^-_{aq} , H-atoms, $OH^\bullet$, H_2O_2) in die oben erwähnten Prozesse involviert.

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