DISSERTATION

Titel der Dissertation

„Electron Emission and Biological Consequences of Hormones in Polar Media, Studied on Testosterone, Progesterone, 17β-Estradiol and Genistein.“

Verfasserin

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DANKSAGUNG

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LIST OF ABBREVIATIONS

16OH DHEA	16-dydroxydehydroepiandrosterone
17OHP	17α-hydroxyprogesterone
17β-HSD	17β-hydroxysteroid dehydrogenase
20αHP	4-pregnen-20α-ol-3-one
2-OHE1	2-hydroxyestrone
3αHP	4-pregnen-3α-ol-20-one
3β-HSD	3β-hydroxysteroid dehydrogenase
4-OHE1	4-hydroxyestrone
5αP	5-pregnane-3,20-dione
A^-	ascorbate
$A^{\bullet}$	ascorbyl radical
AA	L-ascorbic acid
AE	androstenedione
AP-1	activation protein 1
AP-site	apurinic/apyrimidinic site
AR	androgen receptor
ATP	adenosine triphosphate
CEs	catecholestrogens
CYP-450	cytochrome P450
cyt c	cytochrome c
cyt P450scc	cholesterol side-chain cleavage enzyme
DHA	dehydroascorbic acid
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DNA	desoxy-ribonucleic-acid
DSB	double strand break
E. coli	Escherichia coli
E1	estrone
E2	17β-estradiol
E3	estriol
ER	estrogen receptor

G protein	guanine nucleotide-binding proteins
GEN	genistein
GLUT	glucose transporter
Gy	Gray
HBC	(2-Hydroxypropyl)- β-cyclodextrin
HIF1	hypoxia-inducible factor 1
HPLC	high performance liquid chromatography
IκB	inhibitory factor kappa B
JNK	c-Jun N-terminal kinase
MDS	multiply damaged site
MMC	mitomycin C
MOMP	mitochondrial outer membrane permeabilization
mtDNA	mitochondrial DNA
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFκB	transcription nuclear factor kappa B
OD	optical density
p38 MAPK	p38 mitogen-activated protein kinase
P4	progesterone
PR	progesterone receptor
PTP	permeability transition pore
rad	radiation absorbed dose
ROS	reactive oxygen species
SHBG	sex hormone binding globulin
SOD	superoxide dismutase
SSB	single strand break
SVCT	Na+-AA cotransport system
T	testosterone
vitC	vitamin C
vitE	vtamin E
wsE2	water soluble 17β-estradiol
wsP4	water soluble progesterone
β-car	β-carotene

1. ABSTRACT

In recent years, a rapid increase of breast and prostate cancer incidence was observed in the Western countries. Besides lifestyle factors like nutrition *etc.*, sexual hormones and their role in the initiation and progression of cancer development became a centre of interest, especially the action of their individual metabolites. Recently, it was proven that the sex hormones 17β-estradiol (E2) and progesterone (P4) are able to eject electrons (e^-_{aq}) in polar media, when excited in their singlet state. The originating hormone transients are subsequently forming metabolites, some of which can initiate neoplastic processes. Therefore, it is of interest to investigate other sex hormones in this respect and to examine the fate of the resulting transients.

Now, testosterone (T) is found for the first time to eject electrons in a solvent mixture of 60% ethanol and 40% triply-distilled water, when excited to its singlet state by UV-irradiation with monochromatic light of 254 nm wavelength. The phytoestrogen genistein (GEN), which contributes to the comparatively low breast cancer incidence in Asian countries, emits electrons from its excited singlet state, as well. The resulting hormone products can likewise eject e^-_{aq}, but with lower quantum yields of solvated electrons, $Q(e^-_{aq})$, as can be seen by the observed 2nd and 3rd maxima of electron emission. Due to the formation of hormone associates, $Q(e^-_{aq})$ is decreasing with increasing concentration of hormones. As T and GEN are able to emit and to consume electrons, they are classified as "electron mediators".

Comparing the electron emission of T and P4, $Q(e^-_{aq})$ from T turns out to be 3.6 times higher. This fact is due to the different molecular structures of the hormones at position 17 of ring D: T carries a hydroxyl group favouring the electron emission, and P4 a carbonyl group, which consumes a part of the emitted e^-_{aq} from T, leading to a partial regeneration of T. Using vitamin C (vitC) as representative for potent electron donors, it is further shown by high performance liquid chromatography (HPLC) that hormone transients resulting from electron emission can be partly regenerated, if they are still in "status nascendi", by transfer of electrons from an electron donor. T can be regenerated to 58.6% by vitC, which is calculated from the obtained initial quantum yields. Furthermore, the degradation of T is delayed in the presence of vitC. As a consequence of the regeneration of hormones, a decreased formation of carcinogenic metabolites is expected.

In experiments *in vitro* using *Escherichia coli* (*E. coli*) bacteria as a model, the effect of the simultaneously generated oxidizing and reducing free radicals on the metabolites of

different hormones is simulated. Saturation of the bacteria containing medium with various gases before γ-irradiation allows the production of certain concentrations and kinds of primary free radicals as a result of water radiolysis: in aerated medium 46% $OH^{\bullet}$ and 54% $O_2^{\bullet-}$, in medium saturated with N_2O 90% $OH^{\bullet}$ and 10% $H^{\bullet}$, and in medium with argon 44% e^-_{aq}, 10% $H^{\bullet}$ and 46% $OH^{\bullet}$. Hereby, water-soluble E2 (wsE2) with incorporated 2-hydroxypropyl-β-cyclodextrin (HBC) is found to be a very powerful scavenger of $OH^{\bullet}$ and $O_2^{\bullet-}$ radicals. Under the attack of reducing species (e^-_{aq}, $H^{\bullet}$) wsE2 exhibits strong anti-proliferative properties. Water-soluble P4 (wsP4) with incorporated HBC and mixtures of wsE2 and wsP4 exhibit contrary effects compared to wsE2, indicating that a combination of wsP4 and wsE2 may strongly reduce the number of carcinogenic metabolites of wsE2.

T, vitC and their mixture are investigated in a medium containing 4×10^{-2} mol/L ethanol, which has a strong scavenging ability for all primary free radicals except e^-_{aq}. However, in aerated media oxidizing species are operating, whereas in media saturated with argon or N_2O reducing species predominate. T intermediates resulting from attack of oxidizing free radicals show weak cytotoxic properties, but under reducing conditions, T induces strong proliferation of *E. coli* bacteria. These effects are both intensified, when T is combined with vitC.

The present results offer a deeper insight in the biological behaviour of sex hormones and their metabolites. Possible reaction mechanisms of hormones and free radicals are presented, but further radiobiological investigations are required, as the reaction mechanisms are very complicated and overlap with many other biological processes. The pulse radiolysis method could essentially contribute to the elucidation of these processes. The combination experiments with vitC might show new approaches for the application of hormones in medical therapies.

2. ZUSAMMENFASSUNG

In den letzten Jahrzehnten wurde ein rascher Anstieg von Brust- und Prostatakrebsfällen in den westlichen Ländern beobachtet. Neben Lifestyle-Faktoren wie Ernährung usw., rückten auch immer mehr die Sexualhormone und ihre Rolle in der Entstehung und Entwicklung von Karzinomen ins Zentrum des wissenschaftlichen Interesses, hierbei v.a. die spezifische Wirkung ihrer Metaboliten. In diesem Zusammenhang wurde kürzlich erstmals nachgewiesen, dass Sexualhormone wie 17β-Östradiol (E2) und Progesteron (P4) in der Lage sind, in polarem Medium Elektronen zu emittieren, wenn sie in ihrem Singlett-Zustand angeregt werden. Die dabei entstehenden Hormontransienten führen daraufhin zur Bildung von Metaboliten, von denen manche neoplastische Prozesse einleiten. Daher ist es von großem Interesse, weitere Sexualhormone in dieser Hinsicht zu erforschen und das Verhalten der resultierenden Transienten zu untersuchen.

Im Rahmen dieser Arbeit wurde erstmals gezeigt, dass das Sexualhormon Testosteron (T), gelöst in 60% Ethanol und 40% dreifach destilliertem Wasser, auch Elektronen emittiert, wenn es in seinem Singlett-Zustand angeregt wird. Die Anregung erfolgte hierbei durch UV-Bestrahlung mit monochromatischem Licht mit einer Wellenlänge von 254 nm. Ebenso wurde für das Phytohormon Genistein (GEN) gezeigt, welches ursächlich mit dem relativ niedrigen Auftreten von Brustkrebs in Asien in Verbindung gebracht wird, dass es in der Lage ist, Elektronen zu emittieren. Die aus der Elektronenabgabe resultierenden Hormonprodukte konnten ebenso Elektronen emittieren, aber mit geringerer Quantenausbeute an solvatisierten Elektronen ($Q(e^-_{aq})$), wie aus den gefundenen zweiten und dritten Maxima der Elektronenemissionskurven festzustellen war. Interessanterweise verminderte sich $Q(e^-_{aq})$ mit steigender Hormonkonzentration, was auf die Bildung von Hormonassoziaten zurückzuführen ist. Da T und GEN dazu fähig sind, sowohl Elektronen zu emittieren als auch aufzunehmen, wurden sie als „Elektronenmediatoren" klassifiziert.

Im Vergleich der Elektronenemission von T und P4 stellte sich heraus, dass $Q(e^-_{aq})$ von T 3,6mal höher ist. Eine Erklärung hierfür liegt in der unterschiedlichen Molekularstruktur der beiden Hormone: Während T eine Hydroxylgruppe an Position 17 von Ring D trägt, welche die Elektronenemission begünstigt, ist bei P4 an gleicher Stelle eine Karbonylgruppe, welche einen Teil der emittierten Elektronen von T aufnimmt. Hierdurch kam es zu einer partiellen Widerherstellung von T. In weiterer Folge wurde

anhand von Vitamin C (vitC), welches ein gutes Beispiel für einen starken Elektrondonor darstellt, mithilfe der HPLC (Hochleistungsflüssigkeitschromatographie) gezeigt, dass die aus der Elektronenemission resultierenden Hormontransienten zumindest teilweise regeneriert werden können durch Elektronentransfer, sofern sie sich noch in ihrem „Status nascendi" befinden. Hierbei konnte aus den erhaltenen anfänglichen Quantenausbeuten berechnet werden, dass unter den gegebenen experimentellen Bedingungen T zu 58,6% durch vitC regeneriert werden kann. Desweiteren wurde festgestellt, dass sich der Abbau von T in Gegenwart von vitC erheblich verzögert. Durch die Regeneration von Hormonen wird erwartet, dass sich nur vermindert krebserregende Metaboliten bilden.

Die Auswirkung von oxidierenden und reduzierenden freien Radikalen auf Hormonmetaboliten wurde mithilfe von *In vitro* Experimenten mit *Escherichia coli* Bakterien simuliert. Durch die Sättigung der Bakterien enthaltenden Lösungen mit verschiedenen Gasen vor der γ-Bestrahlung konnten definierte Konzentrationen und Arten von freien Radikalen als Produkte der Wasserradiolyse hergestellt werden: In lufthältigem Medium waren dies 46% $OH^{\bullet}$ und 54% $O_2^{\bullet-}$, in N_2O-gesättigtem Medium 90% $OH^{\bullet}$ und 10% $H^{\bullet}$ und luftfreiem Medium (gesättigt mit Argon) 44% e^-_{aq}, 10% $H^{\bullet}$ und 46% $OH^{\bullet}$. Im Rahmen der Experimente wurde festgestellt, dass wasserlösliches E2 (wsE2), welches als Komplex mit 2-Hydroxypropyl-β-cyclodextrin (HBC) vorliegt, ein sehr wirksamer Fänger von $OH^{\bullet}$ und $O_2^{\bullet-}$ ist. Unter dem Einfluss von reduzierenden Radikalen (e^-_{aq}, $H^{\bullet}$) zeigte sich eine starke, antiproliferative Wirkung von wsE2 auf die Bakterien. Konträre Ergebnisse wurden für wasserlösliches P4 alleine (wsP4, im Komplex mit HBC) und im Gemisch mit wsE2 gefunden, was darauf hindeutet, dass eine Kombination der beiden Hormone zu einer erheblichen Reduzierung der karzinogenen Metaboliten von wsE2 führen könnte.

T und vitC wurden auch im Rahmen von *In vitro* Experimenten mit *Escherichia coli* Bakterien untersucht. Allen Medien wurde hierbei 4×10^{-2} mol/L Ethanol zugesetzt, da T nicht in reinem Wasser löslich ist. Da Ethanol ein guter Fänger von allen primären freien Radikalen außer e^-_{aq} ist, waren in lufthältigem Medium nur oxidierende Radikale vorhanden und in Medien gesättigt mit N_2O oder Argon überwiegend reduzierende Spezies. Die aus dem Angriff von oxidierenden Radikalen entstehenden Zwischenprodukte von T zeigten eine schwach zytotoxische Wirkung auf die Bakterien. Unter dem Einfluss von reduzierenden Radikalen induzierte T hingegen starke Proliferation. VitC intensivierte den jeweiligen Effekt von T.

Die erhaltenen Ergebnisse ermöglichen einen tieferen Einblick in das biologische Verhalten von Sexualhormonen und ihren Metaboliten. Mögliche Reaktionsmechanismen von Hormonen mit freien Radikalen werden präsentiert, aber es sind weitere strahlenbiologische Untersuchungen vonnöten, da die Reaktionsmechanismen sehr kompliziert sind und mit vielen anderen biologischen Prozessen überlappen. Die Pulsradiolyse-Methode könnte bedeutend zur Aufklärung dieser Prozesse beitragen. Die Experimente mit Hormon-vitC-Kombinationen könnten neue Ansätze zur Anwendung von Hormonen in der Medizin aufzeigen.

3. INTRODUCTION

3.1. Hormones

The human organism consists of about 10^{14} cells, which can differ in size, compartmentalisation *etc.*, and may be very distant from each other. However, for a coordinated interaction of different cell types of an organism, cell communication is essential. Different types of cell signalling by chemical messengers can be distinguished according to the distance between the communicating cells. If cells generate the signal themselves they are responding to, it is referred to as intern and extern autocrine signalling, respectively. In the latter case, the signal is secreted before it is transduced to the cytoplasm again, whereas in the first case signalling occurs only within the cytoplasm. If the signalling cell is different from the target cell, juxtracrine, paracrine and endocrine signalling can be distinguished. While juxtracrine signalling requires direct cell contact by connexons, the messengers need to diffuse over short distances to adjacent cells after secretion in paracrine signalling. Cells, that are very distant, cannot communicate *via* diffusion-mediated messengers, as diffusion is a very slow process. Therefore, a substance carrying the chemical signal, such as blood, is required. This type of communication is called endocrine signalling.

Hormones represent a class of chemical messengers, which are produced by specialized ductless glands or tissues in very low levels. In general, hormones act as endocrine agents, but cells may also release paracrine or autocrine hormones. Upon binding to specific receptors, which are expressed by the target cell, various effects may be initiated within the target cell through signalling cascades. However, hormones can be distinguished in steroid hormones, peptide/protein hormones and amino acid derivative hormones.

3.1.1. Sex steroids

Sex hormones, such as estrogens, progestogens and androgens, belong to the class of steroid hormones. They are essential for the development of sexual characteristics and behaviour. All sex hormones are derived from cholesterol and share the same core, which consists of four rings (Figure 1). While rings A, B and C are cyclohexanic, ring D consists of solely five carbon atoms. The various sex hormones differ in the oxidative status of the rings and in their attached, functional groups.
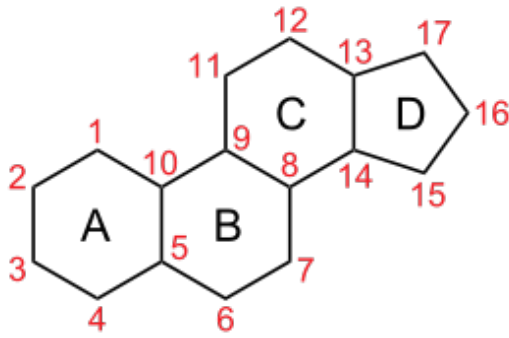
Figure 1. The structure of the four ring core is the same for all sex hormones. Rings are denoted with letters: A, B and C consist of 6 carbon atoms each, whereas ring D is composed of only 5 carbon atoms. The carbon molecules are numbered in a specific manner. (http://en.wikipedia.org/wiki/File:Steran_num_ABCD.svg, 3.2.2011, 18:12)
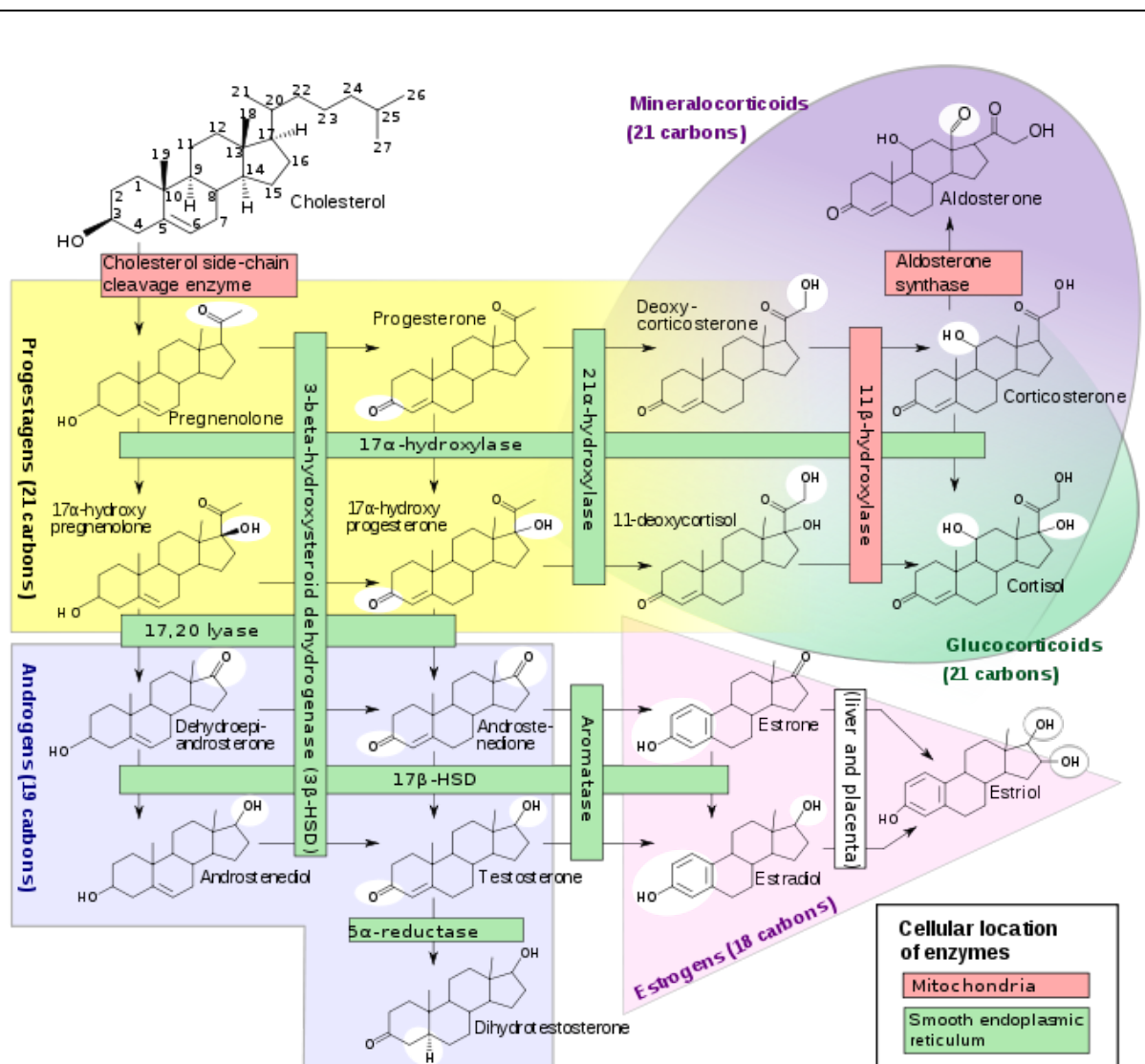
Figure 2. Overview of the steroidogenesis in human beings. Out of the precursor cholesterol (C27), progestogens (C21), androgens (C19), estrogens (C18) as well as mineralo- and glucocorticoids (C21) are synthesized. (http://en.wikipedia.org/wiki/File:Steroidogenesis.svg, 3.2.2011, 18:12)

The pathways of the generation of sex hormones from cholesterol are described by the steroidogenesis (Figure 2). In a first step, cholesterol is converted to pregnenolone by oxidation processes, which are catalyzed by the Cholesterol side-chain cleavage enzyme (cyt P450scc). Further oxidation and a tautomerization process by 3β-HSD (3β-hydroxysteroid dehydrogenase) result in the production of progesterone (P4), the major progestogen. Out of pregnenolone and P4, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (17OHP) can be formed with the help of the 17α-hydroxylase as catalyzing agent. P4 and 17OHP may also be converted to diverse mineralcorticoids by hydroxylation steps.

Androgens are generated from 17α-hydroxypregnenolone and 17OHP by the action of 17,20 lyase, which triggers the splitting off the side chain. In a first step, dehydroepiandrosterone (DHEA) and androstenedione (AE) are produced, which may be further processed to androstenediol and testosterone (T), respectively, a process requiring 17β-HSD. AE and T may also originate from the conversion of DHEA and androstenediol, respectively, through oxidation by 3β-HSD. 5α-dihydrotestosterone (DHT) is produced through reduction of T by the 5α-reductase.

The enzyme aromatase catalyzes the conversion of AE and T to the estrogens estrone (E1) and estradiol (E2). Estriol (E3), the third naturally occurring estrogen is a metabolite of E1 and E2 and for the most part made from sulfonated 16-dydroxydehydro-epiandrosterone (16OH DHEA), which is a derivative of DHEA.

3.1.1.1. Estrogens

The term "estrogens" comprises E1, E2 and their metabolites, which all occur in the human organism, as well as synthetic and other natural substances with estrogenic properties. E2, which is the most biologically potent estrogen (1), is produced by all mammals, females and males, in the gonads, but also in fat cells or the adrenal cortex. Its action in the development of secondary sex characteristics in women (breast development, *etc.*), female reproduction cycle and during pregnancy (placenta development, *etc.*) classifies it as the main female sex hormone. Changes in the body shape affecting bones, joints, fat deposition and structure and skin composition are attributed to E2, as well (2). These effects are primarily initiated at the time of puberty, most enhanced during the reproductive years, and become less pronounced after the menopause, due to an decreasing support with E2. However, E2 is an important factor in

spermatogenesis, too, as it is implicated with the inhibition of male germ cells (3). As sperm counts decrease over the past decades, an association with increasing E2 exposure in the environment is postulated (4).

Within the body, estrogens are metabolised to products, which may either have estrogenic or antiestrogenic activities. In the metabolism of E2, cytochrome P450 (CYP-450) plays a decisive role, as it triggers the hydroxylation processes at position 2 and 4 of ring A, resulting in the formation of the catecholestrogens (CEs) 2-hydroxyestrone (2-OHE1) and 4-hydroxyestrone (4-OHE1), respectively, as well as that of ring D at position 16 (5). Hydroxylation of ring D leads to the generation of a number of metabolites, such as 16α-hydroxyestrone and E3 (Figure 3). 2-OHE1 can be converted into a semichinone, thus, the metabolism of E2 may give rise to free radicals (6), besides the radical scavenging property of E2 due to the phenolic ring A.

In the blood plasma, E2 is bound in large part to SHBG (sex hormone binding globulin) or serum albumin. Only the unbound, free portion may enter a cell and mediate the activation of the target cell receptors ER (estrogen receptor) α and β (7, 8). ERs occur in the cell nucleus or are located to the cytoplasm/cell membrane. Binding of ligands, such as steroid hormones, to nuclear receptors results into ER homo- and heterodimerization, when both ERs are co-expressed. The dimers affect gene transcription by binding to promoters of target genes. The sites of binding to the DNA are called estrogen responsive elements (9). The nuclear estrogen-ER complex may also interact with other transcription factors, such as AP-1 (activation protein 1), and influence their activity (10). Activation of ERs at the plasma membrane mainly results in G-protein (guanine nucleotide-binding protein) mediated signalling (11).

3.1.1.2. Progestogens

Progestogens exhibit pro-gestational functions, as the name implies. P4, the major progestogen, is an important factor in the development of the female mammary gland and is produced during the female menstrual cycle with a peak after ovulation. It causes the growth of the uterus and many other processes, which are necessary for gestation. Also non-reproductive tissues are influenced by P4, such as the cardiovascular or the nervous system (12). In the metabolism of P4 in various tissues, different parts of the molecule are modified (13).
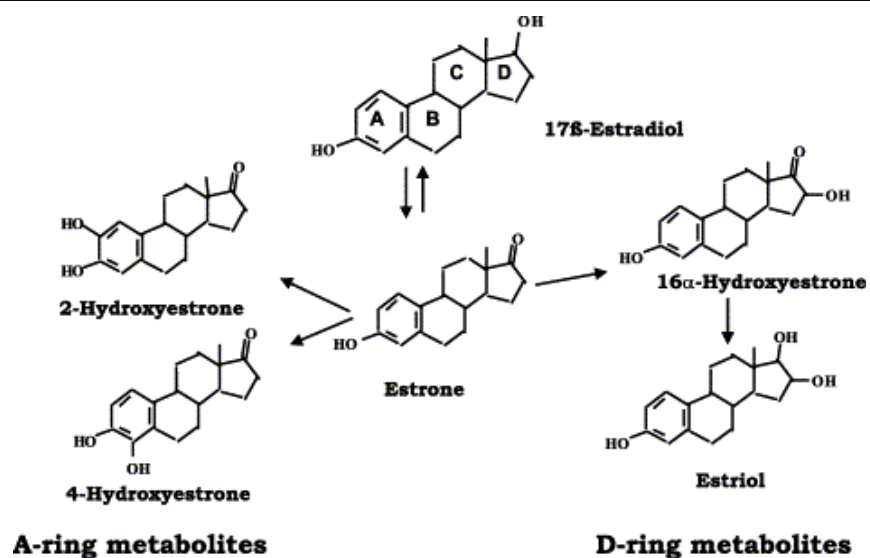
Figure 3. The metabolism of E2 leads to the formation of various A- and D-ring metabolites, which can undergo further degradation (32).
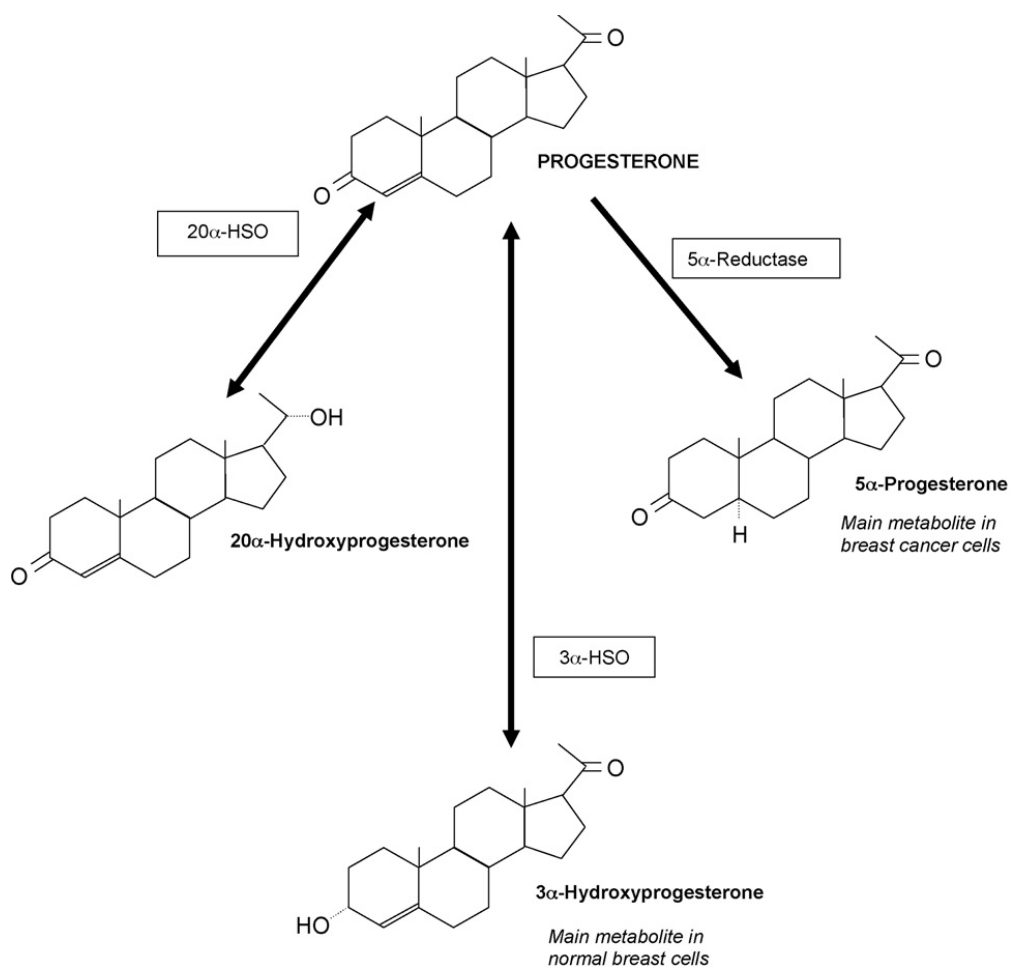
Figure 4. The metabolism of P4 in the breast tissue. 3a-HSO = 3a-hydroxysteroid oxireductase, 20a-HSO = 20a-hydroxysteroid oxireductase (14).

In the breast tissue, P4 is metabolized into two classes of metabolites: 5-pregnane-3,20-dione (5αP) on the one hand, and the 4-pregnen group with 4-pregnen-3α-ol-20-one (3αHP) and 4-pregnen-20α-ol-3-one (20αHP) on the other hand (Figure 4 (14)).

The nuclear receptors of P4 (PR-A and B), which are activated upon ligand-binding, mediate the effect of P4 (15). In the course of this, PR-dimers bind to specific target sequences in gene promoters, which are inducible by P4. Through activation of second messengers and the corresponding signalling cascades, PRs exhibit also non-genotropic effects (16). The effect of P4 in different tissues depends on the specific isoform of PR (PR-A or PR-B), the following nuclear positioning and to a big extent on coregulators (17). Like ER, PR is a member of the nuclear receptor superfamily.

3.1.1.3. Androgens

While the activity of estrogens and progestogens is mainly associated with the development and maintenance of female sex characteristics, androgens are decisive for male features, such as the development of the reproductive tissues (testes, prostate, *etc.*). T is of great importance in females, though: A larger part of androgens than of estrogens is quantitatively produced throughout a woman's lifetime (18). In humans, synthesis of T from AE, which is produced in the adrenal glands in large parts, occurs in the testes (Leydig cells) and ovaries, respectively. T may then be metabolised to DHT, the most potent androgen, in peripheral tissues by the 5α-reductase, or to estrogens. Hence, through this pathway of metabolism, T may initiate estrogenic responses. However, recently it was established, that androgens are also able to specifically bind ERs (19). In both sexes, T was found to act as suppressor of breast growth (20).

Figure 5. Chemical structure of the isoflavonoid genistein compared with 17β-estradiol (after (96)).
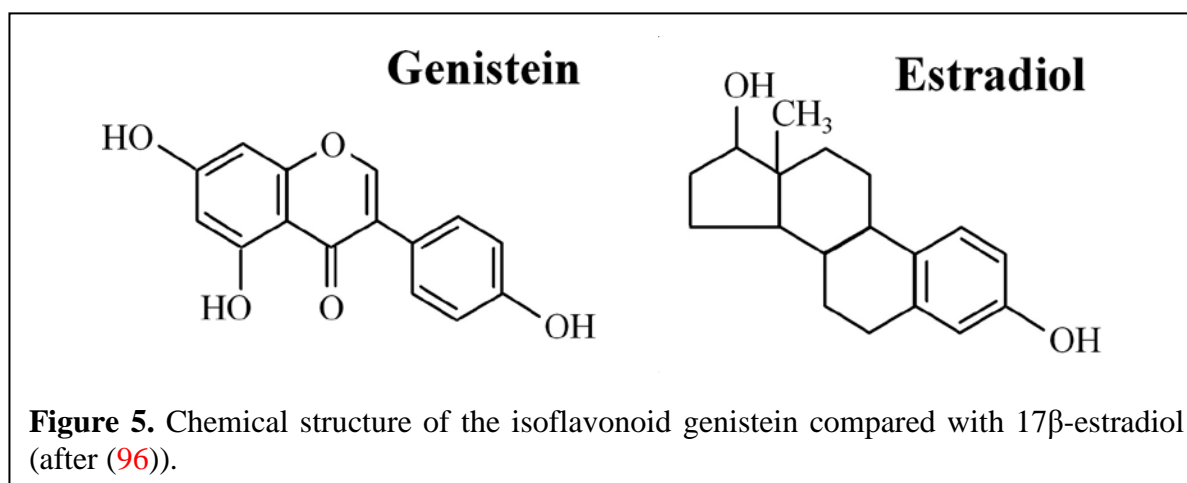
As already mentioned for E2, SHBG and serum albumin are the carriers of T and DHT in the blood stream. Only the unbound fraction of androgens is biologically active. The effect of T and DHT is mediated by the intracellular, ligand-activated androgen receptor (AR), which belongs to the nuclear receptor superfamily (15) and acts as transcription factor on androgen response elements. It is very closely related to PR, and thus, protestogens are able to bind ARs (21). As mentioned above for estrogens and progestogens, a rapid, non-genomic effect of androgens is also proposed (22).

3.1.2. Phytohormone Genistein

Phytohormones, such as isoflavones, gained much interest in the last decades. Basically, phytohormones exhibit important functions in plant development and growth, but upon ingestion by humans with everyday diet, they may be beneficial for health. Isoflavonoids, which are contained in soy, representing a significant portion of the Asian diet, were shown to influence the risk of breast or prostate cancers in Asian countries significantly (23-25). The main isoflavonoid in soy is genistein (4,5,7-trihydroxy-isoflavone, GEN), having a similar molecular structure to E2 (Figure 5) and weak estrogenic properties (101). Thus, it is often referred to as phytoestrogen. GEN is able to bind to both ERs: in comparison with E2, the affinity to ERα is 4% and to ERβ 87% (26). By blocking the ERs for other, more potent estrogens, GEN is able to influence the estrogen metabolism (27). In experiments with MCF-7 cells, GEN was shown to be an antitumor agent, enhancing the efficiency of cytostatica, such as mitomycin C (28). However, GEN is also known to be an antioxidant, that may protect cells against oxidative stress due to its antimutagenic activity (29,30,102).

The reaction rate constants of GEN with various free radicals, which are rather high, were already determined by pulse radiolysis studies, whereby $k(e^-_{aq}+GEN) = 6.2 \times 10^9$ L.mol^{-1}.s^{-1} at pH ~7 (115) and $k(OH+GEN) = 2.3 \times 10^{10}$ L.mol^{-1}.s^{-1} at pH=8.3 (111).

3.1.3. Sex hormones and cancer

It is well known, that lifestyle-factors like nutrition, smoking, alcohol intake, *etc.* have a great impact on the development of various carcinomas. As the incidence for breast and prostate cancer is steadily rising in the Western countries, steroid hormones and their role in the initiation and progression of cancer became a centre of interest. For a long time, the hormone metabolites were thought to be inactive, but now their importance in this respect

is widely accepted within the scientific community and they are more and more subject of studies investigating their impact on various types of cancer cell lines.

For E2 and some of its metabolites, a carcinogenic impact was already demonstrated (31-33,103). E2 and many of its A-ring metabolites like 2-OHE1 and 4-OHE1 show a biphasic pattern concerning cell proliferation: in low concentrations ($1x10^{-8}$ – $1x10^{-6}$ mol/L) they exhibit a stimulating and in high concentrations ($1x10^{-5}$ mol/L and less) an inhibiting effect on cell proliferation. Metabolites of ring D, however, do not show similar results (34). It is postulated that metabolites of E2 might even be more relevant in the induction of breast cancer than E2 itself (104), which implies that the carcinogenity of estrogens may depend firstly on their metabolism and secondly on the resulting composition of metabolites.

Furthermore, estrogens are able to induce genetic instabilities, which is also implicated with the development of cancer (109). Elevated levels of oxidized bases have been found in estrogen induced cancer, and are even thought to precede cancer development. Thus, they may be good biomarkers for cancer risk (110).

Up to now, there is no consensus about the action of P4 on cancer cells. The two kinds of metabolites of P4 in breast tissue (5αP on one hand, and 3αHP and 20αHP on the other hand) exhibit contrary effects: Whilst 5αP promotes mitogenesis and metastasis, 3αHP and 20αHP show none of these attributes (13). Also the 5αP- and E2-modulated increase of ER numbers can be reduced by 3αHP and 20αHP, which underlines the anticancer impact of the 4-pregnen group of the endogenous produced P4 metabolites. Therefore, P4 metabolites seem to act as modulators on ER levels in various ER-positive MCF-7 breast cancer cell lines (35,36).

The role of testosterone in cancer initiation and development is not elucidated yet (37,38). Often, an association between cancer and low or elevated levels of androgens is suggested (39,40). Experimental studies showed both proliferative (41) and anti-proliferative (42) action of testosterone on various carcinoma cell lines. Castagnetta et al. studied the metabolism of T in various prostate cancer cell lines (LNCaP, DU145 and PC3) and found divergent patterns in this respect: While PC3-cells degradated T very quickly to high levels of AE, the other two cell lines didn't convert T to a large part (43). The steroid receptor status of the cell line highly determinates the catalytic preference of the steroid metabolism, which can be either oxidative or reductive (43,44). The same was established for estrogens (45,46).

3.1.3.1. *Sex hormones act as electron mediators*

Recently it was found by Getoff *et al.* (47) that E2 and P4 can eject solvated electrons (e^-_{aq}), when they become electronically excited in their singlet state (cf. chapter 3.2.2. "Solvated electrons"). In the meantime, the same behaviour was shown for various other hormones, such as 4-OHE1 (48), 17OHP (49) and E1 (50). However, E2 is able to eject much more electrons than P4, the rate of yield is two orders of magnitude higher. A possible explanation is the difference of the molecular structures of the two hormones. Taking previously published papers into consideration (51,52), the π-electron structure of ring A and the OH-group at position 3 of E2 are mainly responsible for the observed rather high quantum yields of solvated electrons, $Q(e^-_{aq})$. The resulting phenoxyl-type hormone radical exists in several mesomeric structures, each of which can give rise to metabolites with pro- or anticancer effects. P4, however, produces a radical cation as a consequence of electron emission (47). This radical cation is supposed to be a strong oxidizing species; hence, it can react with various compounds in the cell. During its regeneration with water, OH$^\bullet$ radicals are produced (eq. 1).

$$\textbf{P4}^{\bullet+} \ + \ \textbf{H}_2\textbf{O} \ \rightarrow \ \textbf{P4} \ + \ \textbf{H}^+ \ + \ \textbf{OH}^\bullet \quad \text{(47)} \tag{1}$$

By means of electron transfer from an appropriate electron donor (*e.g.* vitC), P4$^{\bullet+}$ can be either regenerated to P4, or it can lead to the formation of metabolites with different biological properties (53).

In electron emission studies, the polarity of the media was proven to be of great importance. As steroid hormones are insoluble in water, solvents are often mixtures of ethanol and water with varying ratios. Thereby, it was found that with increasing water content, the quantum yields of solvated electrons rose, as well. Furthermore, it was stated that with increasing hormone concentration (1×10^{-5} mol/L and more), $Q(e^-_{aq})$ rapidly decreased. Hence, a formation of unstable complexes (associates) was suggested (47).

Simultaneously to the emission of electrons, the hormones were also shown to scavenge e^-_{aq}. Therefore, these hormones are classified as "electron mediators" (47). For E2, the reaction rate constant with solvated electrons in aqueous solutions is known: $k(e^-_{aq} + E2) = 2.7 \times 10^{10}$ L.mol^{-1}.s^{-1} (54).

3.2. Free radicals

A free radical is defined as any species, molecule or atom, which is capable of independent existence and contains at least one unpaired electron, which makes it highly reactive (55). The unpaired electron occupies an orbital by itself. Free radicals can be negatively or positively charged or electrically neutral.

The simplest free radical is atomic hydrogen ($H^{\bullet}$), as it contains only one electron, which, therefore, has to be unpaired.

One possible way of classifying free radicals in aqueous solutions is to divide them into oxidizing ($OH^{\bullet}$, $O_2^{\bullet-}$, *etc.*) and reducing (e_{aq}^-, $H^{\bullet}$, $R^{\bullet}$, *etc.*) free radicals, corresponding to their redox potential. Otherwise, they can be differentiated into various types according to the atom they are derived from, *e.g.* oxygen-centred free radicals (belonging to the reactive oxygen species, ROS), carbon- or hydrogen-centred free radicals.

3.2.1. Reactive oxygen species

The role of oxygen is very adverse in the human organism: Being an essential molecule for the aerobic metabolism, it can induce cell toxicity and damage at the same time. O_2 contains two unpaired electrons of same spin state in its outer shell, *i.e.*, it can be denoted as "bi-radical". While triplet excited state exceptionally represents the ground state of molecular oxygen ($^3O_2$), which makes it rather reactive, singlet oxygen ($^1O_2$) represents the electronically excited state with two anti-parallel electrons in the outer orbit. Singlet oxygen is a meta-stable, but non-radical oxygen species. Its high reactivity compared to that of O_2 is due to the removal of the spin restriction.

During the reduction of O_2 to H_2O, various types of oxygen-centred free radicals are produced, *e.g.* the superoxide anion ($O_2^{\bullet-}$) by one-electron reduction of O_2, which makes it less reactive than O_2, or the hydroxyl radical ($OH^{\bullet}$), which is the most reactive species and very hazardous to biological tissues.

$$O_2 \ + \ H^{\bullet} \rightarrow HO_2^{\bullet} \tag{2}$$

$$O_2 \ + \ e_{aq}^{-} \rightarrow O_2^{\bullet-} \tag{3}$$

$$HO_2^{\bullet} \ \rightleftharpoons \ H^+ + O_2^{\bullet-} \ (pK = 4.8) \qquad (66) \tag{4}$$

$$HO_2^{\bullet} \ + \ HO_2^{\bullet} \ \rightarrow H_2O_2 \ + \ O_2 \tag{5}$$

$$HO_2^{\bullet} + \ OH^{\bullet} \rightarrow H_2O \ + \ O_2 \tag{6}$$

Table 1. Overview of the most important reactive oxygen species (ROS) in humans.

Reactive Oxygen Species			
Radicals		**Non-radicals**	
$HO_2^\bullet/O_2^{\bullet-}$	Superoxide radical	H_2O_2	Hydrogen peroxide
$OH^\bullet$	Hydroxyl radical	O_3	Ozone
$ROO^\bullet$	Peroxyl radical	$^1O_2$	Singlet oxygen
$NO^\bullet$	Nitric oxide	$ONOO^-$	Peroxynitrite
$RO^\bullet$	Alkoxyl radical	$HOCl$	Hypochlorous acid
$CO_3^{\bullet-}$	Carbonate radical	$HOBr$	Hypobromous acid
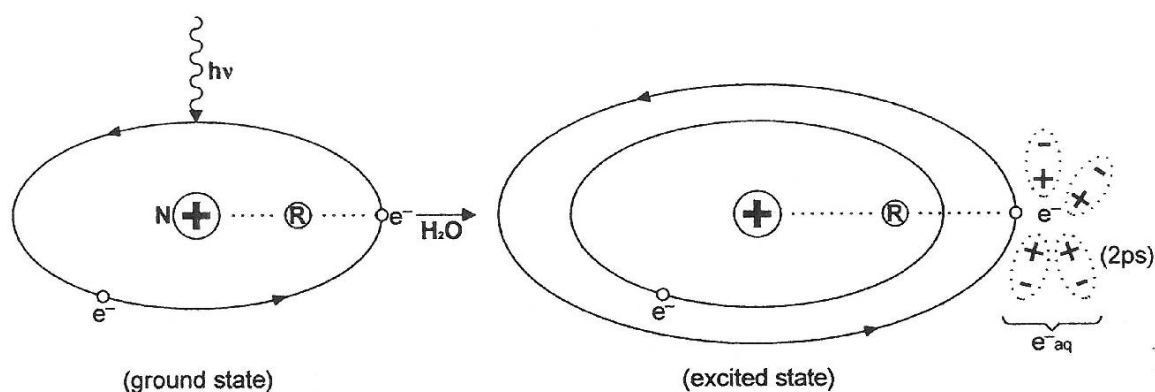
Figure 6. Excitation of biological substances in ground state leads to the ejection of solvated electrons far below the corresponding ionization potential.

S_0......... ground state
S_1, S_2..... singlet excited states
T_1, T_2.....triplet excited states
IC.........internal conversion
ISC.......intersystem crossing
RL........radiationless processes
F..........fluorescence
Ph.........phosphorescence
e^-_{aq}........electron ejection
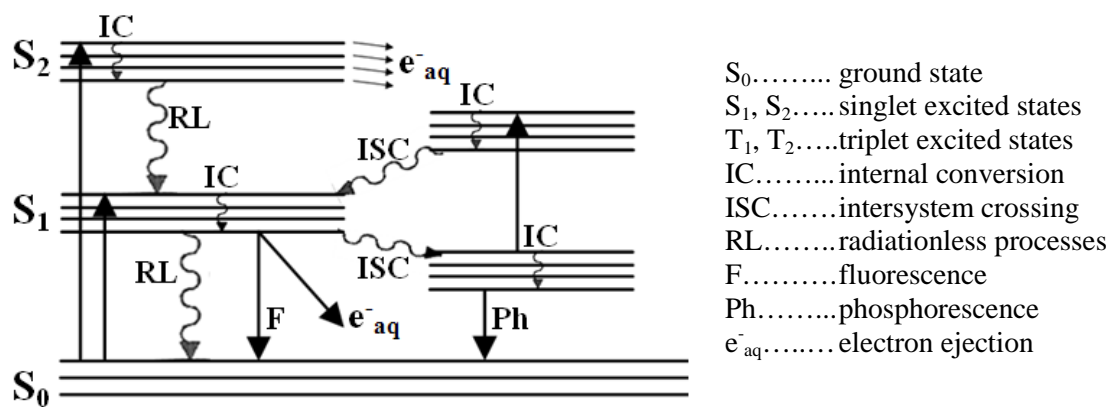
Figure 7. The modified Jablonski diagram illustrates the energy levels of a molecule and the possible transitions (after (52)).

Table 1 comprises the most important ROS, including both oxygen free radicals and some non-radicals (56). Cells have developed a number of defense methods against ROS and their damaging effects, *e.g.* enzymes, anti-oxidants or repair mechanisms, as survival is of prime importance. However, ROS also exhibit beneficial actions within the cell. Therefore, a basic level of ROS is present in the cytoplasm all the time.

3.2.2. Solvated electrons

In 1960, the conversion of γ-irradiated, aqueous CO_2 into simple organic compounds proved the existence of the solvated electrons (e^-_{aq}) for the first time (57). Similar results were obtained by UV-irradiation of aqueous solution containing Fe^{2+} ions, acting as electron donors, and CO_2 as electron acceptor (pH ~3.7) (57,58). In 1962, the absorption spectra of solvated electrons in water and in various polar liquids were measured by pulse radiolysis (59).

The emission of solvated electrons (e^-_{aq}) is a consequence of energy input to a biological molecule (Figure 6). By giving energy to the atom, an electron of the outer orbital is elevated from ground state into the first singlet excited state. Thus, the distance of the e^- to the nucleus is enlarged and the binding energy (F) is reduced with the square of distance. Consequently, the dipoles of the surrounding water molecules become orientated to the polarized atom in less than 10^{-12} s. The e^- is scavenged by the orientated dipoles of the surrounding water molecules and separates from the atom, becoming a solvated electron (e^-_{aq}) with a salvation shell. It should be noted, that the electron emission from excited molecules in their singlet state occurs far below the corresponding ionization potential of a given substrate, because of the very strong dipole action of the water molecules (60).

The quantum yield of e^-_{aq}, $Q(e^-_{aq})$, emitted from a specific substance depends on several factors: molecular structure, substituents like -OH, -OH$^-$, -OPO$_3$H$_2$, -OPO$_3$H$^-$, -COO$^-$,-NH$_2$, -NHCH$_3$, *etc.,* pH of the media and temperature of the solution (52). The ejection of electrons from molecules in singlet state is competing with other photophysical processes, as electronically excited molecules tend to return in their original ground states. The strong relationship between $Q(e^-_{aq})$ and the quantum yield of fluorescence, Q_F, can be taken as example in this respect (52). This is illustrated by the modified Jablonski diagram (Figure 7).

3.2.2.1. *Simulation of emission of e^-_{aq} by UV-irradiation of hormones*

The formation of e^-_{aq} (solvated electrons) is simulated by irradiation of various organic compounds, such as hormones, with monochromatic UV-light in aqueous, air-free solution at pH ~7.4. The basic processes induced by UV-light are shown in eqs. 7 and 8.

$$AB + h\nu \longrightarrow AB^* \diagdown\hspace{-0.5em}\longrightarrow A^\bullet + B^\bullet \quad \text{(formation of radicals)} \quad (7)$$
$$\text{(Excitation)} \qquad\qquad\qquad \text{photophysical processes} \qquad\qquad (8)$$
$$\text{(fluorescence, phosphorescence, } etc. \text{)}$$
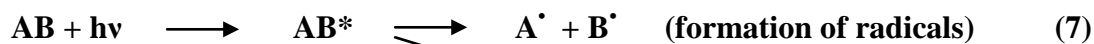
As can be seen in Figure 8, the higher energy portion of the UV-spectrum (overcoming the ionization potential) is considered to be ionizing, whereas the lower energy portion is not. Since the experiments in this study are conducted with a UV-lamp emitting light of λ=254 nm (E=4.85 eV/hν), hormones are just excited in their singlet state and not ionized.

As scavenger for the emitted solvated electrons chloroethanol can be used (eqs. 9,10).

$$ClC_2H_4OH + e^-_{aq} \rightarrow Cl^- + {}^\bullet C_2H_4OH \quad (k = 4\times10^8 \text{ L.mol}^{-1}.s^{-1}, 92) \quad (9)$$
$$Q(Cl^-) = Q(e^-_{aq}) \tag{10}$$

The resulting yield of Cl^- is determined by mercury(II)thiocyanate method (61):

$$2Cl^- + Hg(SCN)_2 \rightarrow HgCl_2 + 2SCN^- \tag{11}$$
$$4Cl^- + Hg(SCN)_2 \rightarrow HgCl_4^{2-} + 2SCN^- \tag{12}$$
$$SCN^- + Fe_3+ \rightarrow Fe(SCN)_2^+ \tag{13}$$
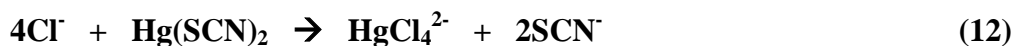
3.2.3. Formation of free radicals by ionizing radiation

Basically, radicals are formed by loosing or gaining a single electron from a non radical, resulting into radical cation and anion, respectively (eqs. 14,15).

$$X - e^- \rightarrow X^{\bullet+} \quad \text{(single electron oxidation)} \tag{14}$$
$$Y + e^- \rightarrow Y^{\bullet-} \quad \text{(single electron reduction)} \tag{15}$$

Radicals can also be formed by homolytic fission (62), a process requiring significant amounts of energy (*e.g.* heat, UV-light, ionizing radiation). Thereby, one electron of the
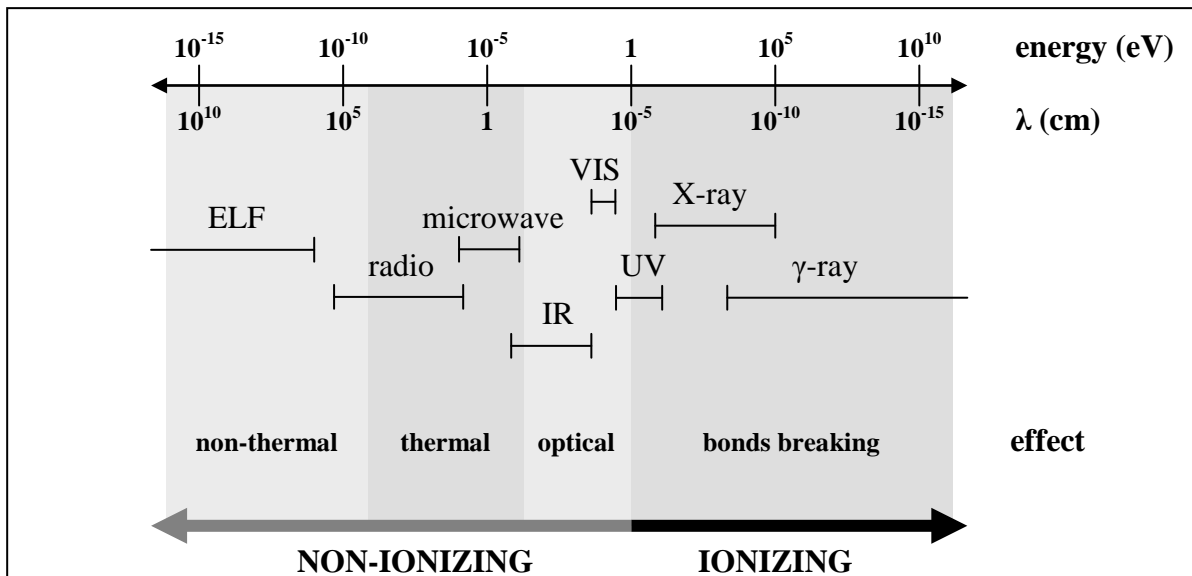
Figure 8. Types of radiation in the electromagnetic spectrum. λ = wave length; ELF = extremely low frequency; IR = infrared; VIS = visible light; UV = ultraviolet.
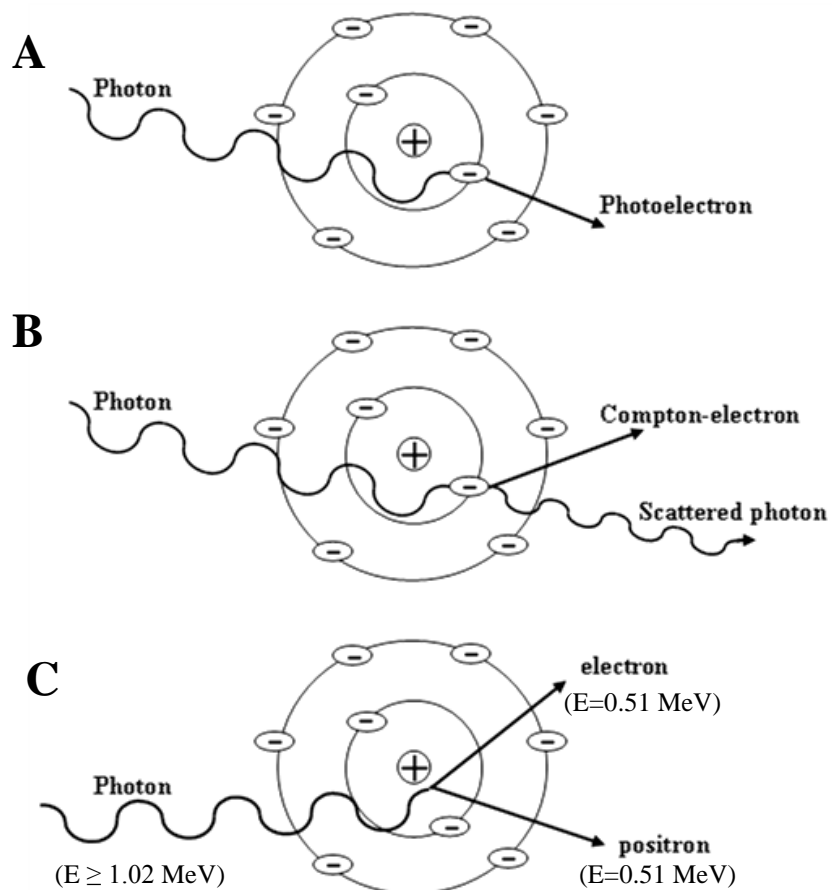
Figure 9. The absorption of γ-ray proceeds according to (A) Photoelectric effect, (B) Compton scattering and (C) Pair production.

bonding pair remains on each atom after cleavage of the covalent bond, and, therefore, two radicals are formed (eq. 16). The opposite of homolytic fission is heterolytic fission, in which one atom receives both electrons after a covalent bond cleavage. Hence, they are ionized, but neither species is a free radical (eq. 17).

$$AB \rightarrow A^{\bullet} + B^{\bullet} \tag{16}$$

$$AB \rightarrow A^{-} + B^{+} \tag{17}$$
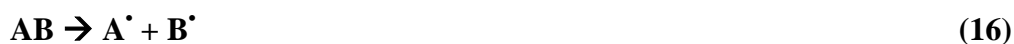
For the generation of oxidizing ($OH^{\bullet}$, $O_2^{\bullet-}$, *etc.*) and reducing (e^{-}_{aq}, $H^{\bullet}$, $R^{\bullet}$, *etc.*) free radicals as products of water radiolysis in the experiments *in vitro* with *Escherichia coli* bacteria (AB1157), ionizing radiation is used. The human organism, consisting of 65-70% water, permanently generates and consumes the same types of free radicals. The production of ionizing radiation for scientific research mainly occurs with ^{60}Co-gamma-rays, and much less by X-ray tubes or electron accelerators.

Ionizing radiation represents the high frequency portion of the electromagnetic spectrum (Figure 8). There are three processes, by which ionizing radiation (X- and γ-ray) interacts with matter: photoelectric absorption, Compton scattering and pair production (Figure 9). They are predominantly dependent on the photon energy of the absorbed radiation and features of the matter.

Photoelectic absorption (photo-effect) occurs, when photons with energies lower than 0.5 MeV encounter atoms with high atomic numbers. All the energy is given to an electron, which subsequently ejects (Figure 9A). If the incident photon has energy from 0.5 to 1 MeV, Compton scattering is predominant. Occuring mainly with atoms of low atomic numbers, it is the most important type of interaction, when water or aqueous solution are irradiated. Hereby, the energy of the incident photon causes the ejection of electrons. The residual energy leads to the emission of a lower energy photon (Figure 9B). Incident photons with energies more than 1.02 MeV can also lead to the production of an electron-positron pair with equal energy of 0.51 MeV (Figure 9C) by converting the energy of the incident photon into the mass of the electron-positron pair according to Einstein's mass-energy equivalence formula. The positron may annihilate by reaction with an electron, resulting in the formation of highly energetic γ-photons. The principal result of all three types of energy transfer mechanisms is the production of excited (eq. 18) or ionized molecules and energetic electrons (eq. 19).

$$AB \xrightarrow{\text{\Large\lightning}} AB^* \longrightarrow A^\bullet + B^\bullet \quad \text{(formation of radicals)} \quad (18a)$$

$$\text{(Excitation)} \searrow \text{photophysical processes} \quad (18b)$$
$$\text{(fluorescence, phosphorescence etc.)}$$

$$AB^+ + e^- \quad \text{(ionization)} \quad (19)$$
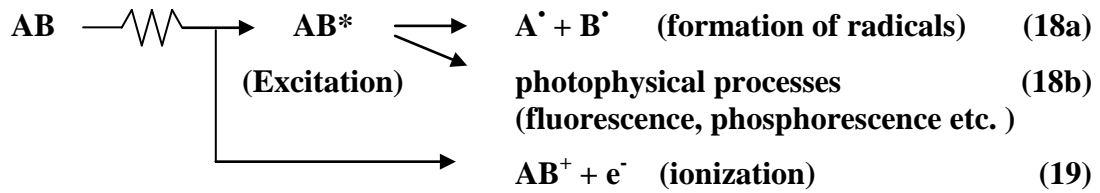
All the time, organisms are exposed to ionizing radiation from the environment (*e.g.* cosmic rays, radioactive decay of natural radioactive substances) and from man-made sources (medical x-ray, *etc.*), on average to 2.4 mSv per year, according to the WHO. "Sievert" (Sv) is the unit of the equivalent dose, which is a measure of the biological effects associated with an exposure to ionizing radiation by applying appropriate weighting factors, according to the different types of ionizing radiation. As different types of ionizing radiation lead to different degrees of tissue damage, it's impossible to infer the likely biological effect just by knowing the absorbed radiation dose. However, the absorbed radiation dose is specified as the amount of energy deposited per unit of mass. The SI-unit of the absorbed radiation dose, "Gray" (Gy), is defined as the absorption of one joule of radiation energy by one kilogram of matter. The old unit "rad" (radiation absorbed dose) is 1/100 Gy.

$$1 \text{ Gy} = 100 \text{ rad} = 1 \text{ J/kg} = 10^4 \text{ erg/g} = 6{,}24 \times 10^{15} \text{ eV.g}^{-1} \quad (20)$$

3.2.3.1. ^{60}Co as irradiation source

In this study, γ-radiation was implemented (Figure 8). The source of γ-radiation is Cobalt-60 (^{60}Co), a radionuclide undergoing radioactive decay and emitting γ-rays and β-particles. ^{60}Co is produced by exposing natural cobalt (^{59}Co) to thermal neutrons in a reactor (eq. 21).

$$^{59}\text{Co} + \text{n} \rightarrow {}^{60}\text{Co} + \gamma \quad (21)$$

This reaction leads to an unstable ^{60}Co-nucleus. ^{60}Co decays with a half life of 5.26 years by the process of ß-emission with an energy of 0.308 MeV to an excited state of ^{60}Ni. The emission of ß-particles is easily shielded and the particles are mainly absorbed within the Co-source itself. The generated ^{60}Ni-nucleus immediately emits two γ-rays with energies of 1.17 MeV and 1.33 MeV in order to reach a stable state of ^{60}Ni. With time, the activity of ^{60}Co decreases according to eq. 22.

$$A = A_0 * e^{-kt} \tag{22}$$

A……...activity

A_0……. activity at time 0

k……... radioactive decay constant

t……… time

Several methods exist in order to determine the activity of a γ-source (ionization chamber, calorimeter, *etc.*). During this study, the dose-rate of the γ-source was determined and permanently controlled by means of a Fricke-Dosimeter (63), which was subsequently modified by saturation with oxygen. Hereby, Fricke solution is exposed to ionizing radiation. The resulting primary products of water radiolysis oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) in the presence of air. The increase of Fe^{3+} can be measured spectrophotometrically at λ=305 nm (eqs. 23-25). It should be noted, that temperature strongly affects the molar extinction coefficient (ε_{305}) of ferric ions, as ε_{305} is proportional to the temperature of the dosimeter solution (105).

$$Fe^{2+} + HO_2^{\bullet} + H^+ \longrightarrow Fe^{3+} + H_2O_2 \tag{23}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \tag{24}$$

$$Fe^{2+} + OH^{\bullet} \longrightarrow Fe^{3+} + OH^- \tag{25}$$

3.2.3.2. *Radiolysis of water and aqueous solutions*

When water is exposed to ionizing radiation, it decomposes producing a large number of primary products: free radicals and molecular products. The energy is deposited in 10^{-15} s in the media, resulting in the production of excited (eq. 26) and ionized (eq. 27) water molecules.

$$H_2O \longrightarrow H_2O^* \longrightarrow H^{\bullet} + OH^{\bullet} \tag{26}$$

$$H_2O^{\bullet +} + e_s^- \longrightarrow e_{th}^- \longrightarrow e_{aq}^- \tag{27}$$
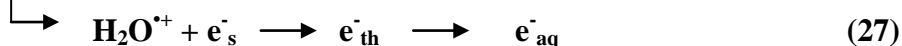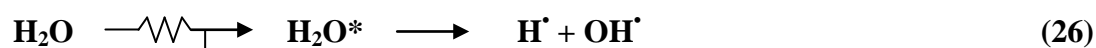
Excited water molecules can decompose to $H^{\bullet}$ and $OH^{\bullet}$ radicals or transfer their energy to other molecules (energy transfer process). After deceleration of the electrons to thermal energy (~10^{-14} s) because of interactions with the media, they are captured by water through dipolar interactions (eq. 27). This process called solvatation takes ~$2x10^{-12}$ s in water: The electron is now called a solvated electron (e_{aq}^-) and surrounded by several

water molecules. The number of water molecules is dependent on the temperature. Simultaneously, radical cations ($H_2O^{\bullet+}$) react with water molecules in 10^{-13} s (eq. 28) and result in the formation of OH radicals and hydroniumions ($H_3O^+ = H^+_{aq}$).

$$H_2O^{\bullet+} + n\ H_2O \longrightarrow OH^\bullet + H_3O^+ \qquad (28)$$

In less than 10^{-12} s after the absorption of the ionizing radiation, the generated species are non-homogenously distributed in spurs along the track, *i.e.* centers of high concentrations of free radicals. These species can undergo intra-spur reactions (Table 2), as they start diffusing, resulting into molecular products, such as H_2 or H_2O_2. Transients, which are existent at the time of homogenously distribution throughout the bulk of the solution (after $<10^{-7}$ s) are called the primary products of water radiolysis. The gross reaction of water radiolysis and the radiochemical yields of the primary products (G values) in the pH-range of 6 to 8.5 are given in eq. 29.

$$H_2O \longrightarrow\!\!\wedge\!\!\wedge\!\!\longrightarrow\quad e^-_{aq},\quad H^\bullet,\quad OH^\bullet,\quad H_2,\quad H_2O_2,\quad H^+_{aq},\quad OH_{aq} \qquad (29)$$
$$\text{(G value at pH 6-8.5)}\quad (2.7)\quad (0.6)\quad (2.8)\quad (0.45)\quad (0.72)\quad (3.2)\quad (0.5)$$
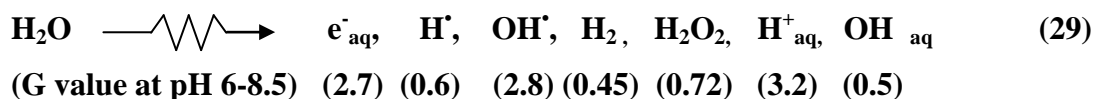
The radiochemical yield (G value) is a measure for the chemical effect of ionizing radiation on a substrate, simply giving the number of produced or consumed species per 100 eV absorbed energy. For conversion into SI-units, the G value has to be multiplied by 0.10364 in order to obtain G(X) in μmol.J^{-1}. Initial G values (G$_i$) are calculated before back reactions take place.

The pH of the solution plays a critical role in the G values (Figure 10), as $H^\bullet$ is the conjugate base of e^-_{aq} (eqs. 30,31). At high pH $OH^\bullet$ and H_2O_2 (eqs. 32,33) dissociate (64,65). However, the total radical yield within the pH-range of 6 to 8.5 is G = (OH + H + e^-_{aq}) = 6.1 = 0.632 μmol.J^{-1} absorbed energy.

Low pH	$e^-_{aq} + H^+ \rightarrow H^\bullet$	(30)
	$H^\bullet + OH^- \rightarrow e^-_{aq}$	(31)
High pH	$OH^\bullet \rightleftharpoons O^- + H^+ \qquad$ (pK = 11.9)	(32)
	$H_2O_2 \rightleftharpoons H^+ + HO_2^- \qquad$ (pK = 11.7)	(33)
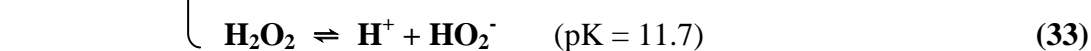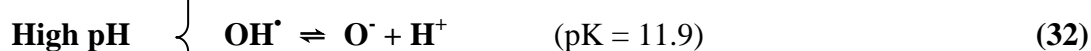
Table 2. Radiolysis of water and reactions of the primary species with the particular rate constants (92, 65).

Primary reactions:

$$H_2O \rightsquigarrow \begin{cases} H_2O^* \longrightarrow H + OH \\ H_2O^+ + e^-; \quad e^- \longrightarrow e_{th}^- + n\,H_2O \longrightarrow e_{aq}^- \\ \quad\quad\quad\quad H_2O^+ + H_2O \rightarrow H_3O^+ + OH \end{cases}$$
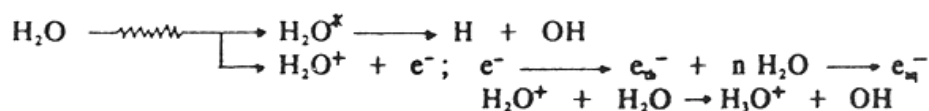
Gross reaction of water radiolysis (the G-values) at pH 7 are given in brackets)*:

$$H_2O \rightsquigarrow e_{aq}^-, \quad H, \quad OH, \quad H_2, \quad H_2O_2, \quad H_{aq}^+, \quad OH_{aq}^-$$
$$\quad\quad\quad (2.7) \quad (0.6) \quad (2.8) \quad (0.45) \quad (0.7) \quad (3.2) \quad (0.5)$$

Major primary reactions:

$H_{aq}^+ + OH_{aq}^- \longrightarrow H_2O$	($k = 1.4 \times 10^{11}$ dm³mol⁻¹s⁻¹)
$H + H \longrightarrow H_2$	($k = 1.0 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$H + OH \longrightarrow H_2O$	($k = 2.5 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$H + e_{aq}^- \longrightarrow H_2 + OH_{aq}^-$	($k = 2.0 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$OH + OH \longrightarrow H_2O_2$	($k = 6.0 \times 10^{9}$ dm³mol⁻¹s⁻¹)
$OH + e_{aq}^- \longrightarrow OH_{aq}^-$	($k = 2.5 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$e_{aq}^- + e_{aq}^- \longrightarrow H_2 + 2\,OH_{aq}^-$	($k = 3.0 \times 10^{9}$ dm³mol⁻¹s⁻¹)
$e_{aq}^- + H_{aq}^+ \longrightarrow H$	($k = 2.3 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$H + OH_{aq}^- \longrightarrow e_{aq}^-$	($k = 2.5 \times 10^{7}$ dm³mol⁻¹s⁻¹)

$$OH \rightleftharpoons H_{aq}^+ + \dot{O}_{aq}^- \quad\quad (pK = 11.9)$$
$$H_2O_2 \rightleftharpoons H_{aq}^+ + HO_{2\,aq}^- \quad\quad (pK = 11.65)$$

In the presence of oxygen:

$H + O_2 \longrightarrow H\dot{O}_2$	($k = 2.1 \times 10^{10}$ dm³mol⁻¹s⁻¹)
$e_{aq}^- + O_2 \longrightarrow \dot{O}_2^-$	($k = 1.9 \times 10^{10}$ dm³mol⁻¹s⁻¹)

$$H\dot{O}_2 \rightleftharpoons H^+ + \dot{O}_2^- \quad\quad (pK = 4.8)$$

In the presence of N₂O:

$e_{aq}^- + N_2O \longrightarrow OH + OH^- + N_2$	($k = 0.91 \times 10^{10}$ dm³mol⁻¹s⁻¹)

In the presence of H₂:

$OH + H_2 \longrightarrow H_2O + H$	($k = 3.5 \times 10^{7}$ dm³mol⁻¹s⁻¹)

---

*) G-value = number of changed molecules per 100 eV (1.60×10^{-17} J) absorbed energy.
For conversion into SI-units: multiply the G-value by 0.10364 to obtain G(x) in μmol.J⁻¹.
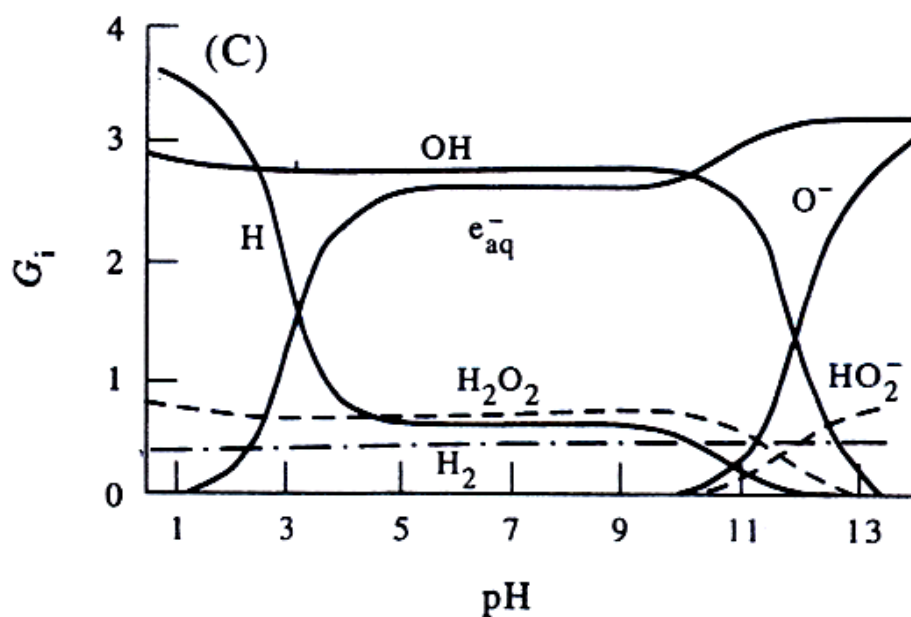
Figure 10. G_i values of the primary products of water radiolysis as a function of pH (64, 65).
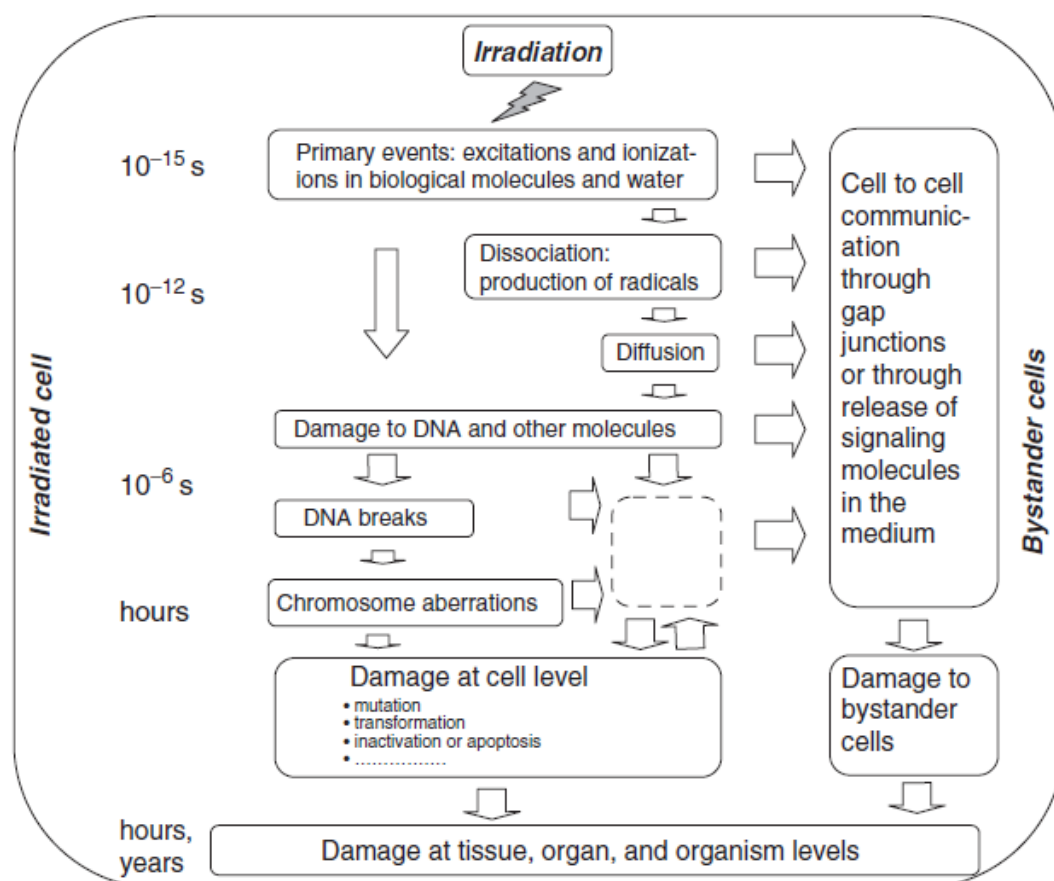
Figure 11. Incident ionizing radiation leads to damages at various levels of the irradiated cell. Unirradiated bystander cells can also be affected (72).

In order to obtain specific types of free radicals, solutions are saturated with different gases. In air-free media (saturation *e.g.* with argon), the acting primary species are 46% $OH^{\bullet}$, 44% e^{-}_{aq} and 10% $H^{\bullet}$. In the presence of air, totally oxidizing conditions can be achieved. All reducing primary species are converted into oxidizing $HO_2^{\bullet}$ / $O_2^{\bullet-}$ (eqs. 34-36), resulting into 46% $OH^{\bullet}$ and 54% $O_2^{\bullet-}$ at pH ~7.4.

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet} \tag{34}$$

$$e^{-}_{aq} + O_2 \rightarrow O_2^{\bullet-} \tag{35}$$

$$HO_2^{\bullet} \rightleftharpoons H^{+} + O_2^{\bullet-} \quad (pK = 4.8) \qquad (66) \tag{36}$$
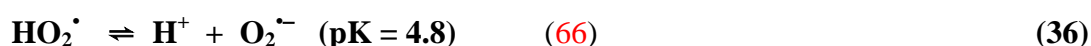
In media saturated with N_2O, e^{-}_{aq} are converted into $OH^{\bullet}$ radicals (eq. 37), resulting into 90% $OH^{\bullet}$ and 10% $H^{\bullet}$.

$$e_{aq} + N_2O \rightarrow OH^{\bullet} + OH^{-} + N_2 \quad (k = 0.9x10^{10} \text{ L.mol}^{-1}.s^{-1} \text{ (92))} \tag{37}$$
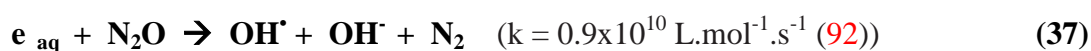
In aqueous, air-free media containing ethanol, all primary free radicals except e^{-}_{aq} are scavenged by ethanol and converted into reducing ethanol radicals (eqs. 38,39 (67)).

$$OH^{\bullet} + C_2H_5OH \rightarrow {}^{\bullet}C_2H_4OH + H_2O \qquad (k = 2x10^{9} \text{ L.mol}^{-1}.s^{-1} \text{ (92))} \tag{38}$$

$$H^{\bullet} + C_2H_5OH \rightarrow {}^{\bullet}C_2H_4OH + H_2 \qquad (k = 1.7x10^{7} \text{ L.mol}^{-1}.s^{-1} \text{ (92))} \tag{39}$$
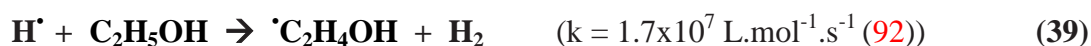
Simultaneously, the radiolysis of ethanol leads to formation of solvated electrons and several organic free radicals (67). Thus, in airfree media the acting free radicals are mainly e^{-}_{aq} and ${}^{\bullet}C_2H_4OH$, both reducing species. In aerated solutions, the ethanol radicals react with oxygen, resulting in the formation of oxidizing peroxyl ethanol radicals (eq. 40 (66)).

$$CH_3{}^{\bullet}CHOH + O_2 \rightarrow CH_3CH(O_2^{\bullet-})OH \tag{40}$$

3.2.3.3. *Biological action of ionizing radiation*

Since cells consist to a high percentage of water, it is most likely, that radiation interacts predominantly with water molecules and less with other cellular components (indirect radiolysis). As described above, a number of free radicals and other primary

species result from the radiolysis of water, which start diffusing throughout the solution. In doing so, it is possible, that besides reacting among themselves, they react with other substances present in the solution (*e.g.* the components of a cell). That indirect action of ionizing radiation is the predominant process when radiating with gamma rays, which are defined as low LET radiation. LET (linear energy transfer) is a measure for the energy transferred to a specific material penetrated by an ionizing particle of specified energy per unit distance traversed. Gamma-rays are highly penetrating and lose their energy less effectively than *e.g.* α- and β-rays.

If ionizing radiation interacts with substances of a cell others than water, this process is referred to as direct effect of ionizing radiation. This process is predominant with high LET radiation. The excited or ionized atoms of biological compounds may undergo intra- or intermolecular energy transfer processes or/and intramolecular electron transfer processes ([106]), which means that the absorbed energy is able to migrate within the molecule or even between two molecules. The site of damage can therefore be different from the site of the interaction with ionizing radiation.

Cell damages, which are induced by ionizing radiation, may lead to mutations or loss of functions of DNA, proteins or other important cell compounds and to cell death. In *E. coli*, the greater part of lethal damage is due to indirect biological action of ionizing radiation ([68,69]). Since there is a correlation between radiation sensitivity and amount of DNA in a cell ([70]), it is expected, that eukaryotic cells are much more sensitive towards ionizing radiation than *E. coli* bacteria, which were implemented in the radiation studies. Furthermore, the radiosensitivity of cells is highly dependent on the specific phase of cell cycle ([71]).

As DNA is always hydrated by water molecules due to its negative charge, it is one of the main targets for cell damage by ionizing radiation ([72]). Regarding the rate constants for the primary products of water radiolysis and DNA (for $OH^\bullet$: $k=0.3 \times 10^9$ $dm^3.mol^{-1}.s^{-1}$; for $H^\bullet$: $k=0.08 \times 10^9$ $dm^3.mol^{-1}.s^{-1}$; for e_{aq}: $k=0.14 \times 10^9$ $dm^3.mol^{-1}.s^{-1}$) and the corresponding G values, it can be assumed, that the $OH^\bullet$ radicals contribute to a large part to the damage ([70]). Radiation-induced lesions in DNA include single strand breaks (SSB), double strand breaks (DSB), base alterations or loss (apyrimidinic / apurinic (AP) site), tandem or clustered lesions as well as cross-links (DNA/DNA or DNA/protein cross-links). The same lesions are results of radiotherapy ([73]). Hereby, DNA repair is essential for the organisms, as non-repaired DNA damage usually causes cell death due to

the inhibition of necessary biological processes, such as replication or transcription. A combination of the different lesions in close vicinity on the DNA strand (multiply damaged site (MDS)) leads to much more harm to cells than single damage events, as the repair machinery is much more challenged (74). Since DNA repair is not error-free, mutations can occur, which are not lethal but may be harmful and a possible initiator of carcinogenesis.

In Figure 11 the biological effects of ionizing radiation are depicted at different spatial and time levels (72). Interestingly, cells may suffer from radiation damage, which have never been hit by ionizing radiation, but were in close vicinity to irradiated cells (75, 76). These bystander effects are due to cell-cell communication between irradiated and unirradiated cells.

3.2.4. Free radicals in the human organism – only a burden?

For a long time, free radicals were solely associated with damage within the scientific community. Nowadays, there is a large body of evidence, that oxidizing ($OH^{\bullet}$, $O_2^{\bullet-}$, *etc.*) and reducing (e^-_{aq}, $H^{\bullet}$, $R^{\bullet}$, *etc.*) free radicals play a determining role in a huge number of biological processes in the human organism. Moreover, both types, oxidizing as well as reducing free radicals, fulfill equally important tasks in living systems.

In the human organism, the generation of free radicals can be induced by exogenous inducers, such as UV-light, tobacco smoking *etc.* However, a variety of endogenous sources is producing free radicals as well, such as the generation of ROS in the mitochondrion. Thereby, it is essential, that the production of the radicals is well regulated, as an increased production of ROS exhibits hazardous effects on cells, resulting in DNA-damage, carcinogenesis and initiation of a number of diseases (diabetes mellitus, atherosclerosis, neurodegenerative diseases, *etc.*) by influencing intercellular signaling cascades or direct oxidation of cellular key-components (79).

3.2.4.1. Generation of free radicals in the mitochondria

In mitochondria, a good portion of endogenous ROS is produced as side-products of the respiratory chain. The ubisemiquinone, which is part of the electron transport chain producing ATP, is, together with 8 other sites, responsible for the generation of superoxide radical anion species by univalent reduction of O_2 (77). By dismutation, $O_2^{\bullet-}$ is

converted into H_2O_2. Free Fe(II) and other metals in the cytoplasm promote the Fenton and Haber-Weiss reaction, leading to the formation of $OH^{\bullet}$ out of $O_2^{\bullet-}$ and H_2O_2.

If detoxification of the ROS by antioxidants or enzymes is not possible due to an imbalance between ROS production and antioxidant defenses, the mitochondrion suffers from oxidative stress. As ROS are highly reactive species, they immediately attack nearby compounds (nucleic acids, membrane lipids, *etc.*), trying to gain an electron. The damaged compounds may not be longer able to carry out their function, resulting into mitochondric dysfunction by mutation or even apoptosis / necrosis by changing the permeability of the membranes (Figure 12). However, the presence of ROS is also beneficial, as they play a significant role in signal transduction processes (78,79).

Figure 12. The mitochondrial ROS production is the main source for reactive species in cells. Cyt c: cytochrome c; MOMP: mitochondrial outer membrane permeabilization; mtDNA: mitochondrial DNA; PTP: permeability transition pore; ROS: reactive oxygen species (97).
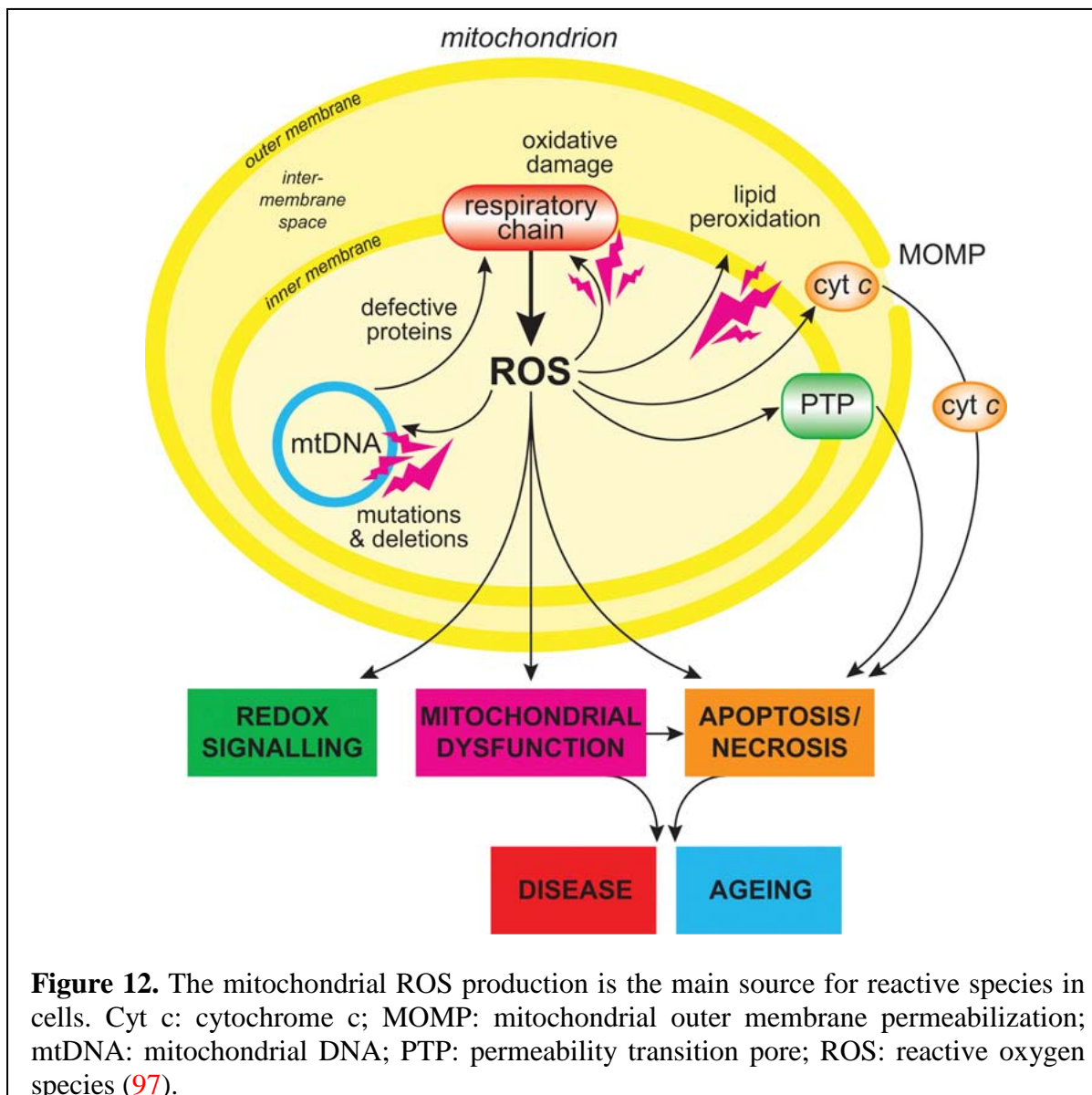
3.2.4.2. Free radicals as regulatory mediators in cell signaling

Oxidative stress has long been thought to be unregulated and an unwanted side-effect of the aerobic metabolism, as it is involved in various pathological processes. Nowadays, it becomes more and more evident, that free radicals are of crucial importance in a number of signalling processes of the cell, mediating diverse biological activities, such as inflammation or vasodilatation (78). Modulations of cell transduction, which are initiated by free radicals through specific modifications of cell signalling proteins, are referred to as „redox cell signalling" (79). Groundbreakingly was the prove, that NFκB, an important transcription factor for genes of the early defence system, is activated by oxidizing reagents, such as H_2O_2, and even ionizing radiation (80,81). The activation of NFκB is a result of an increase of the degradation of its inhibitor IκB by ROS. Generally, it can be stated, that oxidative attack on signalling molecules either results into a loss or gain of function or into a function switch. Meanwhile, a great deal of signalling molecules regulated by free radicals has been identified and described. Besides the already mentioned NFκB, the transcription factor AP-1, p38 MAPK and JNK, just to name a few, seem to be tightly regulated by free radicals (79).

3.2.4.3. Intracellular killing of bacteria by phagocytes

Phagocytosis is a process, in which phagocytes (macrophages and neutrophils) engulf foreign particles or bacteria and enclose it into a phagosome. After formation of the phagocytic vacuole by fusion of the phagosome with a lysosome, ROS are generated in order to kill the internalized bacterium (82). Hereby, the phagocytic NADPH-oxidase, which assembles in the membrane of the lysosome, catalyzes the synthesis of the superoxide radical ($O_2^{\bullet-}$) by transferring two electrons from NADPH across the membrane to O_2 (eq. 41).

$$\text{NADPH} - 2e^- + 2O_2 \rightarrow \text{NADP}^+ + \text{H}^+ + 2O_2^{\bullet-} \tag{41}$$

As oxygen uptake is remarkably increased by this reaction, this process is called "oxidative burst" or "respiratory burst". This process in not associated with cellular respiration, though. However, the superoxide radical can be converted by the superoxide dismutase (SOD) into H_2O_2 and singlet oxygen. Subsequently, $O_2^{\bullet-}$ and H_2O_2 may form OH$^\bullet$ and again singlet oxygen. These ROS altogether induce lethal damages to the internalized bacterium, and are, therefore, crucial for the immune response.

Figure 13. Biologically active states of vitamin C (88).

Figure 14. Overview of the cascade electron transfer between the antioxidant vitamins C, E and β-carotene in the cell membrane (53).
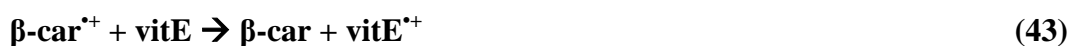
3.3. Antioxidants

Aerobe organisms cope with the poisonous action of oxygen by a complex system of antioxidant defense mechanisms. Basically, an antioxidant is any substance that reduces or retards oxidation of an oxidizable substrate (56). There are several processes, by which antioxidants can protect the cell from damage, whereby the first line of defense is the prevention of the formation of ROS and other free radicals. This can be done by shielding important key-substance with the help of chaperones, offering alternative targets for the attack of free radicals (56) or by decreasing the availability of pro-oxidants, *e.g.* Fe(II), which triggers the Fenton and Haber-Weiss reaction (83). If free radicals are already produced, it is necessary to inhibit chain reactions. Diverse enzymes, such as SOD or catalase, or proteins, *e.g.* gluthathione, vitamin C (vitC) or vitamin E (vitE), react with free radicals in various manners. A large part of the antioxidants becomes oxidized themselves upon reaction with free radicals by acting as electron donors.

3.3.1. Vitamin C

One of the most potent antioxidants is L-ascorbic acid (AA, vitC, Figure 13), which is a water-soluble reducing agent and a very potent electron donor. Its name derives from scorbut (scurvy), a disease resulting from nutritional deficiency of AA, which leads to defects in collagen synthesis (84). AA is synthesized as product of the hexuronic pathway in the liver or kidney by the gunololactone oxidase in many organisms. As humans are deficient of this enzyme, they need to obtain AA by nutrition. Therefore, AA is classified as vitamin in humans, which is ubiquitously distributed in the cells upon absorption in the intestine. Depending on the target cells, AA is either actively transported by sodium-ascorbate co-transporters (SVCTs), or it has to be oxidized to dehydroascorbic acid (DHA) for transportation by hexose transporters (GLUTs) (85). In the latter case, DHA is reduced to AA in the cytoplasm again.

The antioxidant properties of AA can be demonstrated by the cascade electron transfer (Figure 14), by which lipoproteins of membranes are protected from free radical attack (53). It is known that cell membranes consist to approximately 5% of vitamins C, E and β-carotene. However, attacking free radicals are reduced in a first defense line by β-carotene, which is located at the outer membrane layer (eq. 42). The ejected electron from β-car neutralizes the oxidizing species and becomes oxidized itself to a radical cation (β-car$^{\bullet+}$), which in turn is regenerated by vitE (eq. 43).

$$\text{OH/ROO}^{\bullet} + \beta\text{-car} \rightarrow \text{OH-/ROO-} + \beta\text{-car}^{\bullet+} \tag{42}$$

$$\beta\text{-car}^{\bullet+} + \text{vitE} \rightarrow \beta\text{-car} + \text{vitE}^{\bullet+} \tag{43}$$

The radical cation ($E^{\bullet+}$) is subsequently regenerated to vitE by ascorbate (A^-) (eq. 44), which presents a very low reduction potential and is oxidized to an ascorbyl radical ($A^{\bullet}$). The relatively stable ascorbyl radicals can dismutate to AA and DHA (eq. 45), terminating the chain reaction.

$$\text{vitE}^{\bullet+} + \text{A}^- \rightarrow \text{vitE} + \text{A}^{\bullet} \tag{44}$$

$$2\text{ A}^{\bullet} + \text{H}^+ \rightarrow \text{AA} + \text{DHA} \quad (86) \tag{45}$$
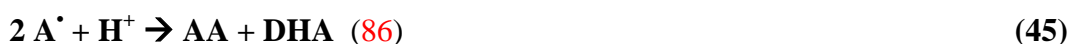
Interestingly, vitC can also act as pro-oxidant by reducing redox-active metals, such as Fe^{3+} or Cu^{2+}, triggering the Fenton reaction, which results in the formation of $OH^{\bullet}$ or lipid peroxidation (87). The inhibitory effect of vitC on cancer cells seems to be partly due to this process producing reactive species, as cancer cells tend to overexpress GLUTs and, therefore, accumulate more vitC in the cytoplasm than normal cells (88). Furthermore, cancer cell lines exhibit a higher basal, endogenous level of reactive species. Hence, a substance like vitC, which is promoting the production of reactive species, will challenge tumor cells much more than normal cells (89).

In tumors, hypoxia occurs, a phenomenon due to the rapid growth, leading to regions within the tumor, which are deprived from oxygen. Tumor cells usually adapt to hypoxia by activation of HIF1 (hypoxia-inducible factor 1), but AA inhibits this process and, thus, acts as anticancer agent (90).

Under ionizing irradiation, AA shows a protective effect on *E. coli* bacteria, especially in aerated solutions (91). The reaction rate constants for A^- with some primary species of water radiolysis were measured by pulse radiolysis at pH ~7: $k(OH^{\bullet}+A^-) = 1.1\times10^{10}$ $dm^3.mol^{-1}.s^{-1}$, $k(H^{\bullet}+A^-) = 3\times10^8$ $dm^3.mol^{-1}.s^{-1}$ and $k(e^-_{aq}+A^-) = 3.5\times10^8$ $dm^3.mol^{-1}.s^{-1}$ (92). DHA alone and in combination with vitE or/and β-car essentially enhances the antitumor effect of the cytostaticum mytomycin C (MMC) (107).

4. STUDY OBJECTIVES

In order to get a deeper insight in the rather complicated biological processes in the organism, which proceed simultaneously and mostly interact with each other in a harmonious way, the objectives of the present thesis embrace several superimposed problems.

Since the breast and prostate cancer risk is steadily increasing in the Western countries, research focuses more and more on sex hormones and their metabolites, as they exhibit an impact on the carcinogenesis of most breast- and prostate cancer types (31-46). Based on previous knowledge, that biological substances having some functional substituents like -OH, $-OH^-$, $-OPO_3H_2$, $-OPO_3H^-$, $-COO^-$,$-NH_2$, $-NHCH_3$ *etc.*, are able to eject electrons when excited in their singlet state in aqueous solutions (52), it is expected that hormones can principally also emit electrons in polar solvent. The originating hormone transients are subsequently forming metabolites, some of which can initiate neoplastic processes. This supposition was proven firstly for E2 and P4 as representatives of hormones in a mixture of water and ethanol (47). Based on these experimental data and knowledge, it was of biological interest, to examine this subject matter on other hormones, such as the main male sex hormone T or the phytohormone GEN. T and E2 differ in their molecular structures only at ring A, where T carries a carbonyl group and E2 a hydroxyl group, whereas T and P4 differ at ring D, where T carries a hydroxyl group and P4 a carbonyl group. Therefore, comparisons between these three hormones could contribute to a better understanding of the impact of the individual functional groups in this respect. The molecular structure of GEN, which is associated with the comparatively low breast cancer incidence in Asian countries and is known to exhibit weak estrogenic properties (101), is similar to E2, and, thus, also of interest.

It is further postulated, that a reduction of the number of carcinogenic metabolites resulting from hormones after electron ejection can be achieved by the regeneration of hormones with the help of a potent electron donor, due to an electron transfer from the donor to the acceptor. Therefore, using vitC as representative for a potent electron donor and T as acceptor, HPLC-analyses were performed, in order to study the degradation of the substrates under UV-irradiation and a possible regeneration.

In addition to this, it was of great importance to test the obtained results by experiments *in vitro*. In the organism, which consists of 65-70% water, oxidizing ($OH^\bullet$, $O_2^{\bullet-}$, *etc.*) as well as reducing (e^-_{aq}, $R^\bullet$, *etc.*) free radicals are permanently generated,

playing a determining role in a huge number of biological processes. A tight regulation is essential, as a surplus of free radicals may lead to hazardous effects and a great number of possible damages. However, in order to study the effects of oxidizing and reducing species on various sex hormones, experiments *in vitro* with *Escherichia coli* bacteria were performed. As the same types of free radicals are generated by ionizing radiation as products of water radiolysis, it is possible to gain desired kind and concentration of free radicals under appropriate experimental conditions. Since the ability of E2 and P4 to emit electrons when excited in their singlet state was already proven, they were investigated individually and in mixture in this respect first. In the course of these studies, both hormones were embedded in a water-soluble complex with HBC. Thereafter, also experiments *in vitro* with T and vitC were carried out in media containing 4×10^{-2} mol/L ethanol.

Finally, special attempts were made to suggest probable reaction mechanisms explaining the observed chemical and biological effects.

5. MATERIALS AND METHODS

5.1. Hormones and vitamins

In this study, a number of different hormones, phytohormones and vitamins were applied, as can be seen in Table 3, being of highest purity available. Since some of the hormones were not soluble in water or physiological, aqueous media, they were dissolved in a mixture of 40 vol.% triply-distilled water and 60 vol.% ethanol of p.a. purity.

5.2. Sterilization of materials

Before using any glass materials, they were treated in a steam-pressure autoclave for 20 min at 121°C and afterwards cleaned with a glassware washer (Mielabor G 7783 by Miele). Subsequently, the glassware was sterilized by heat (250°C, 6 h).

Plastic materials for the experiments in vitro, such as Petri dishes or Falcon flasks, were obtained as sterilized one-way dishes. Other plastics (plugs, *etc.*) were sterilized by firstly cleaning them with a glassware washer (Mielabor G 7783 by Miele) and secondly irradiating them with γ-rays over night.

Solutions for the experiments *in vitro* with *Escherichia coli* bacteria, such as media and buffer, were prepared in sterilized glass vessels and treated in a steam-pressure autoclave for 20 min at 121°C. Hormones and vitamin C were solved in sterilized buffer and sterile filtrated.

5.3. Sources of radiation

During the experiments, two different kinds of radiation were applied. In the investigations concerning the emission of electrons and HPLC, solutions were irradiated with UV-light in a specially designed apparatus. In the frame of the experiments *in vitro*, ionizing radiation produced by a γ-irradiation source was used.

5.3.1. UV-irradiation apparatus

The double-walled UV-irradiation apparatus with 4π-geometry is shown in detail in Figure 15. A connected thermostat enabled keeping a desired temperature constantly throughout the whole experiment. Solutions could also be saturated with gas.

Table 3. Overview of the hormones, phytohormones and vitamins used in this study.

Substance	Company	Product No.	Quality characteristics	Solvent
Testosterone	Fluka	86500	≥99%	40% triply-distilled water and 60% p.a. ethanol
Progesterone	Sigma	P0130	≥99%	40% triply-distilled water and 60% p.a. ethanol
β-Estradiol-Water soluble	Sigma	E4389	balance HBC: 47 mg E2 per gram	triply-distilled water
Progesterone-Water soluble	Sigma	P7556	balance HBC: 76 mg P4 per gram	triply-distilled water
(2-Hydroxypropyl)-β-cyclodextrin	Sigma	C0926		triply-distilled water
Genistein	Fluka	91955	≥98%	triply-distilled water
L-Ascorbic Acid	Sigma	A5960	≥99%	triply-distilled water
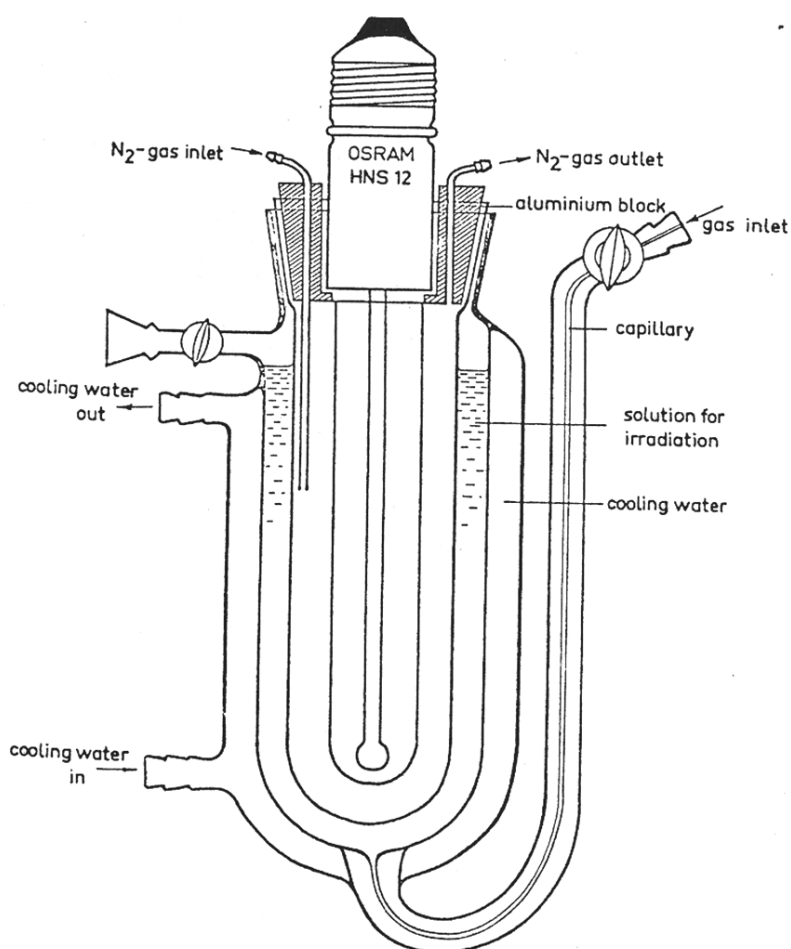
Figure 15. Irradiation apparatus for solutions and gases using a low-pressure Hg-lamp (λ=253.7 nm) with thermostat junctions (108).

As UV-source, a low pressure Hg-lamp (HNS 12, Osram, 12 Watt) with incorporated Vycor-filter was used. Due to the removal of the 184.9 nm line by the filter, the lamp delivered monochromatic light of 254 nm (4.85 eV/hυ).

5.3.1.1. *Monochloracetic Acid Actinometry*

The intensity of the emitted UV-light ($\sim 1 \times 10^{18}$ hυ.ml^{-1}.min^{-1}) was determined by monochloracetic acid actinometer (93,94). As it is dependent on the geometry of the irradiation vessel, it had to be repeated with every vessel being applied.

5.3.1.1.1. Reagents

Reagent A: (50 ml)

0.375 mol Fe(III)-Nitrat in 5.25 mol.L^{-1} HClO4 dissolved in triply distilled water

Reagent B: (50 ml)

Ethanol saturated with Hg(II)-thiocyanate

5.3.1.1.2. Establishment of the calibration curve

Procedure:
- prepare 1 L of 10^{-4} mol.L^{-1} NaCl
- put 0, 1, 3, 6 and 8 ml in a 10 ml flask
- add 1 ml of reagent B and 1 ml of reagent A
- add triply distilled water to a final volume of 10 ml
- after 5 min determine the optical density spectrophotometrically at $\lambda = 460$ nm using triply distilled water as reference and quartz cuvettes
- calculate ΔOD_{460} values of the samples by subtracting the OD_{460} value of the 0 sample from the OD_{460} value of each sample
- create a diagram illustrating the concentration of Cl$^-$ ions versus ΔOD_{460} values

5.3.1.1.3. UV-irradiation of monochloracetic acid

Procedure:
- prepare 250 ml of 1 mol.L^{-1} monochloracetic acid
- put the solution in the UV-irradiation apparatus
- keep the temperature at 37°C for at least 20 min
- take the unirradiated sample (0 sample, ~1 ml) and put 0.2 ml in a 10 ml flask

- add 1 ml of reagent B, 1 ml of reagent A and triply-dist. water to a final volume of 10 ml

- after 5 min determine the optical density spectrophotometrically at $\lambda=460$ nm using triply distilled water as reference and quartz cuvettes

- start UV-irradiation and take samples after 1,2,4 and 6 min and proceed likewise

- calculate ΔOD_{460} values of the samples by subtracting the OD_{460} value of the 0 sample from the OD_{460} value of each sample

- draw a diagram illustrating the duration of UV-irradiation versus ΔOD_{460} values

- calculate the slope of the curve

- calculate I_0 in $h\upsilon.ml^{-1}.min^{-1}$ according to eq. 46:

$$I_0 = k * \frac{N_L}{Q(Cl^-) * 1000} \tag{46}$$

k ...... slope of the curve
N_L ...... Loschmidt number (6.023×10^{23} molecules.mol^{-1})
$Q(Cl^-)$ ...... quantum yield of Cl^-, dependant on the temperature

- multiply the I_0 value by 50 because of the different dilutions in the calibration curve and the irradiation curve

5.3.2. 60Co gamma source

As γ-irradiation source for the generation of free radicals, a ^{60}Co-gamma-irradiator (Gammacell 220, AECL, Nordion Ltd., Canada), providing a dose rate of approximately 30 $Gy.min^{-1}$ was used. The equipment is depicted in Figure 16.

5.3.2.1. *Fricke dosimetry*

The activity of the "Gammacell 220" had to be measured, since ^{60}Co has a half life of 5.26 years. During this study, either Fricke dosimetry was performed, or a computer program based on Fricke dosimetry was used.

Procedure:

- irradiate Fricke solution (10^{-2} mol/L $FeSO_4*7$ H_2O + 10^{-2} mol/L NaCl + 0.8 N H_2SO_4) with ^{60}Co-gamma-rays for 0, 40, 80, 120, 160 and 240 s

- irradiate two samples for the 0 sec sample, i.e. put the solution into the sample chamber and lower, but then retract immediately

- determine the optical density of the irradiated solution spectrophotometrically at $\lambda=305$ nm using non-irradiated Fricke solution as reference
- measure the temperature of the solution
- calculate the molar extinction coefficient of Fe(III) according to Figure 17

Figure 16. ^{60}Co-gamma-irradiation source ("Gammacell 220", AECL, Nordion Ltd., CA).
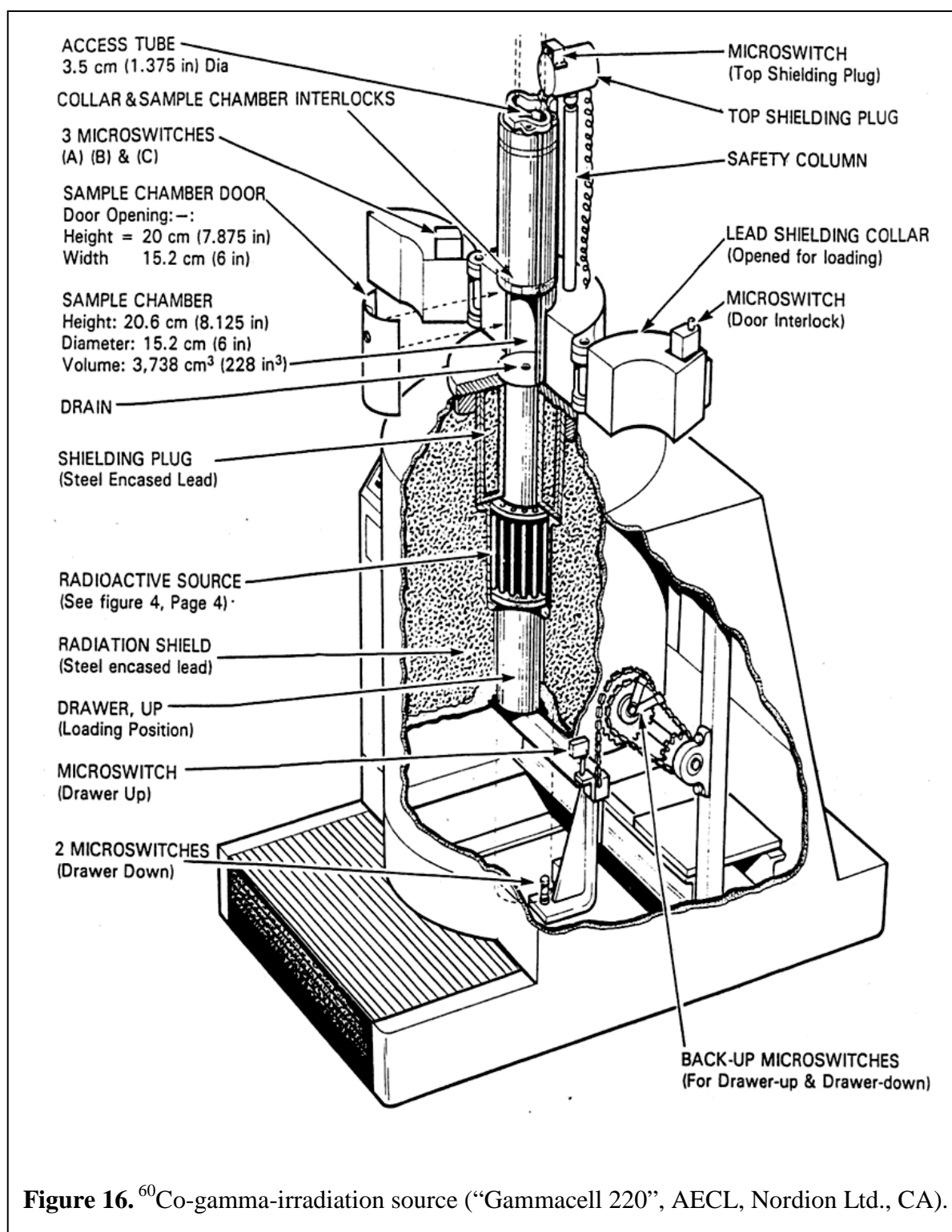
- calculate the absorbed radiation dose (D) in rad according to eq. 47 (95):

$$D = 9.65 * 10^8 * \frac{\Delta OD}{\varepsilon * d * \rho * G(Fe^{3+})} \qquad (47)$$

ΔOD …… difference of the optical density of irradiated and non-irradiated
solutions, whereby
$$OD = \varepsilon * c * d$$
ε …… molar extinction coefficient of Fe(III)
d …… optical pathway
ρ …… density of the solution (1.025 g/ml for Fricke solution)
$G(Fe^{3+})$ ...... chemical yield of Fe^{3+}; is equal to 15.6

- draw a diagram illustrating the duration of irradiation versus D(krad)
- calculate the slope of the curve

5.4. Saturation of solutions with air, N_2O or argon

Saturation of solutions was performed in order to obtain specific kinds and concentrations of free radicals (Table 2, eqs. 34-40). In experiments using UV-light, solutions can be saturated with various gases within the UV-irradiation apparatus (Figure 15) before irradiation. Since this is impossible for samples due to γ-irradiation, the solutions had to be saturated with air, N_2O or argon separately prior to irradiation, in order to obtain specific types of free radicals.

Procedure:
- put samples in a test tubeand seal with flexible film (Parafilm)
- when saturating with air, leave the tube alone
- when saturating with N_2O or argon, penetrate the flexible film by a glass Pasteur pipette
- saturate the solution for 15 min by conducting the gas through the Pasteur pipette
- pull the Pasteur pipettes out and seal the test tubes at once with flexible film
- further procedures according to the different protocols thereafter

5.5. Formation of carboxylic acids

The pH-change of irradiated samples was registered by a pH-meter (PHM 83 – AUTOCAL pH-meter, Radiometer, Copenhagen), indicating the formation of carboxylic acids from *e.g.* irradiated hormones.
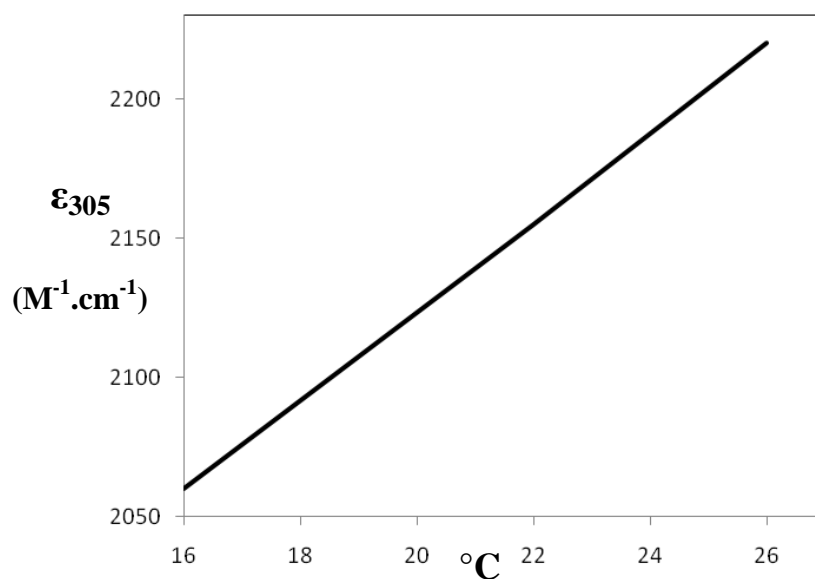
Figure 17. Molar extinction coefficients (ε in $mol.L^{-1}.cm^{-1}$) of Fe(III) ions as a function of temperature (in °C) (105).
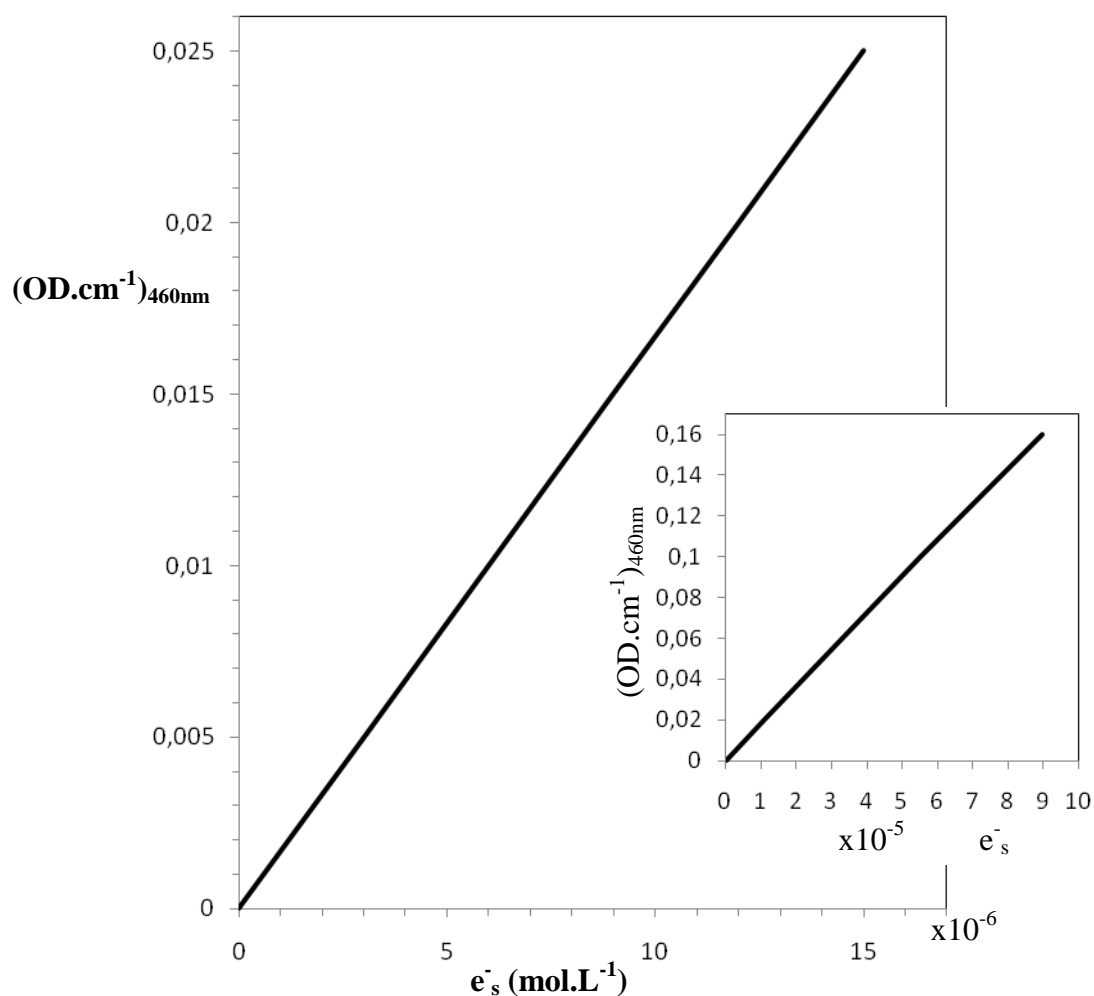
Figure 18. Calibration curve for the determination of the concentration of solvated electrons, when knowing the ΔOD values.

Procedure:

- adjust samples to pH ~7.4 by adding NaOH or HClO₄

- saturate samples with argon, N₂O or air

- irradiate with UV- or γ-rays with various doses

- measure the pH exactly 5 min after irradiation

- calculate ΔpH by subtracting the pH value of the non-irradiated sample from the pH values of the irradiated samples

5.6. Spectrophotometrical measurement of electron ejection

The emission of electrons from hormones was simulated by UV-excitation of hormones in aqueous, air-free solutions (pH~ 7.4, 37°C; cf. chapter 3.2.2.1. "Simulation of emission of e^-_{aq} by UV-irradiation of hormones"). For the registration of absorption spectra and OD values, a LAMBDA 650 Spectrophotometer (Perkin-Elmer) was used, belonging to double beam UV/Vis spectrophotometers with microcomputer electronics.

5.6.1. Reagents

Reagent A: (50 ml)

0.375 mol Fe(III)-Nitrat in 5.25 mol.L^{-1} HClO4 dissolved in triply distilled water

Reagent B: (50 ml)

Ethanol saturated with Hg(II)-thiocyanatef

Chloroethanol:

Applied as delivered

5.6.2. Electron emission

Procedure:

- prepare substrate solution in desired concentration and solvent in a 200 ml flask (saturate the solvent with argon for 20 minutes in advance!)

- measure pH (~7.4) and correct if necessary by adding NaOH or HClO₄

- make an absorption spectrum of the substrate solution from 200-500 nm using the solvent as reference and quartz cuvettes

- check OD value at 254 nm

- put the substrate solution used for the spectrum and the pH measurement back into 200 ml flask

- add $1x10^{-2}$ mol/L ClEtOH to the substrate solution (134 µl)
- put the solution in the UV-irradiation apparatus, saturate with argon for ~30' and adjust the temperature to 37°C
- take sample "0" from the radiation-apparatus (~4 ml) before starting UV-irradiation
- put 1 ml of the sample in a 10 ml flask
- add 1ml of reagent B and 1ml of reagent A and fill up with water
- wait for 5 min before measuring OD at 460 nm
- measure pH of the sample with the remaining 3 ml
- start UV-radiation
- take other samples as required and do as described above
- calculate ΔOD by substracting OD of sample "0" from each sample
- determine the concentration of e^-_{aq} with the help of the calibration curve (Figure 18)
- calculate the number of absorbed quanta, whereby $OD = \log (I_0/I)$ and $I_A = I_0 - I$

5.7. HPLC analyses

The photoinduced degradation of hormones individually and in mixture as well as their regeneration by electron transfer in a mixture with vitamin C was registered by HPLC-method. The implemented apparatus was model Hewlett-Packard / Agilent 1100 HPLC series with a series 1050 diode array detector (DAD), using the Zorbax Eclipse XDB-C18 column (150x4.6mm I.D., 5 µm particle size, Agilent) at a temperature of 30°C.

Testosterone, progesterone and their mixture were separated by eluent A (Table 4), a linear gradient between mobile phases A ($2.5x10^{-4}$ mol/l ammonium acetate in water) and B (acetonitrile). Detection of the injected samples of 15 µl was performed at 244 nm. Total run time was 20 min with a flow rate of 0.3 ml.min^{-1}.

Table 4. Eluents used in HPLC-analyses, both driving a linear gradient between 2 mobile phase: $2.5x10^{-4}$ mol/L ammonium acetate in water (AmmAc) and acetonitrile (ACN).

Eluent A			Eluent B		
min	ACN	0.25 mM AmmAc	min	ACN	0.25 mM AmmAc
0	50	50	**0**	80	20
0.2	60	40	**0.6**	65	35
8	90	10	**24**	10	90
9	90	10	**27**	10	90
9.7	50	50	**30**	80	20

Testosterone, vitamin C as well as their mixture were separated by eluent B (Table 4), a linear gradient between mobile phases A ($2.5x10^{-4}$ mol/l ammonium acetate in water) and B (acetonitrile). Detection of the injected samples of 25 µl was performed at 244 nm for T and 250 nm for VitC. The total run time was 35 min, followed by 5 min of post run with a flow rate of 0.25 ml.min^{-1}.

Procedure:

- prepare substrate solution in desired concentration and solvent in a 200 ml flask (saturate the solvent with argon in advance!)
- measure pH (~7.4) and correct if necessary by adding NaOH or $HClO_4$
- make an absorption spectrum of the substrate solution from 200-500 nm using the solvent as reference and quartz cuvettes
- check OD value at 254 nm
- put the substrate solution used for the spectrum and the pH measurement back into 200 ml flask
- put the solution in the UV-irradiation apparatus
- saturate with argon for ~30' and adjust the temperature to 37°C
- take sample "0" from the radiation-apparatus (~4 ml) before starting UV-irradiation
- put 1 ml of the sample in a vial suitable for the HPLC-apparatus
- measure pH of the sample with the remaining 3 ml
- start UV-radiation
- take other samples as required and do as described above
- calculate ΔOD by substracting OD of sample "0" from each sample
- calculate the number of absorbed quanta, whereby $OD = \log (I_0/I)$ and $I_A = I_0 - I$
- set up the HPLC (computer, pumps, wavelength detector, *etc.*)
- put the samples in the auto-sampler and start the HPLC
- print HPLC chromatograms afterwards and compare the obtained areas under curve for the specific peaks

5.8. Experiments in vitro with *Escherichia coli* bacteria

The gram-negative, facultative anaerobe bacterial strain *Escherichia coli* is very common in the lower intestine of warm-blooded organisms. Due to its comparatively simple handling and genetics, *E. coli* is one of the most favorite model organisms in

biochemical research. The strain used in this study (*Escherichia coli* AB1157; DSM 9036, DSMZ, Braunschweig, Germany) has the following chromosomal markers:

F⁻ *thr-1 ara-14 leuB6* Δ(*gpt-proA)62 lacY1 tsx-33* qsr⁻ *supE44 galK2* lambda⁻ rac⁻ *hisG4*(Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3*(Oc) *thi-1*

5.8.1. Media and buffer

Agar-medium:

10 g/L tryptone, 5 g/L yeast, 10 g/L NaCl, 15 g/L Agar

LB-medium:

10 g/L tryptone, 5 g/L yeast, 10 g/L NaCl

Buffer-solution (pH 7.4):

8 g/L NaCl, 0.2g/L KCl, 0.25 g/L KH_2PO_4, 1,46 g/L $Na_2HPO_4.2H_20$

5.8.1.1. Preparation of Agar Plates

Procedure:

- prepare agar medium (4 L for ~200 plates)
- sterilize the agar medium in the steam-pressure autoclave for 20 min at 121°C
- let it cool down to approximately 65°C
- pour medium in Petri dishes (20 ml per PD, 9 cm diameter)
- treat the surface with flame in order to kill spores
- store the agar plates in the fridge

5.8.2. Rehydration

The bacterial strain *Escherichia coli* AB1157 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and delivered as dried culture. The rehydration was done following the company's instructions.

Procedure:

- open the ampoule in a sterile hood
- add 1 ml LB-medium and allow the pellet to rehydrate for 30 min
- mix

- transfer into a test tube and add 10 ml LB-medium

- streak a drop onto 4 agar plates

- incubate at 37°C o/n

- remove some colonies by using an inoculation loop for further culturing and streak onto new agar plates

- repeat several times, before using the bacteria for experiments

5.8.3. Storage

5.8.3.1. *Long-term storage*

For long time storage (up to 5 years), glycerol stock cultures were prepared by adding 0.85 ml of the bacteria in logarithmic phase in LB-medium to 0.15 ml of the cryoprotectant glycerol. Subsequently, the well-mixed stock cultures were freezed at -20°C.

5.8.3.2. *Short-term storage*

For short term storage (1 month), bacteria were cultivated on an agar plate. Thereby, some colonies were removed from an old agar plate by using an inoculation loop and quadrant streaked on a new agar plate. After an o/n incubated at 37°C, the bacteria were stored at 4°C in a refrigerator.

5.8.4. Radiation-biological analyses

5.8.4.1. *Batch culture*

The density of the bacteria as well as their phase of growth strongly influences the results of experiments. Therefore, it was absolutely necessary to work with a defined concentration of bacteria being in a specific phase of growth throughout all experiments. For this purpose, batch cultures were grown.

Procedure:

- remove some colonies of the agar cultures with a one-way inoculation loop

- inoculate into 6 ml LB-medium and incubate o/n shaking (140 rpm) in a water-bath at 37°C

- in the next morning add 3 ml to 100 ml fresh LB-medium

- incubate on the shaking water bath at 37°C, until the suspension reaches an optical density (OD) of 0.4 at $\lambda=580$ nm, corresponding to an approximately concentration of 1 to 2×10^8 bacteria per ml

5.8.4.2. *Cytotoxicity assay*

In order to establish a suitable concentration of the different hormones (Table 5) for the following experiments under irradiation, a cytotoxicity assay had to be performed. If the substance happened to be insoluble in water, ethanol was used as solvent, whereby the final concentration of ethanol in buffer was always 0.5% per sample (4×10^{-2} mol/L).

Table 5. Hormones and their concentrations, which were tested for their toxicity on *E. coli* bacteria.

wsE2		wsP4		T (+0.5% EtOH)	HBC
C_{E2} (mol.L^{-1})	C_{HBC} (mol.L^{-1})	C_{P4} (mol.L^{-1})	C_{HBC} (mol.L^{-1})	C_T (mol.L^{-1})	C_{HBC} (mol.L^{-1})
0	0	0	0	0	0
1×10^{-6}	3.98×10^{-6}	1×10^{-6}	2.72×10^{-6}	1×10^{-6}	3.98×10^{-6}
5×10^{-6}	1.99×10^{-5}	1×10^{-5}	2.72×10^{-5}	5×10^{-6}	1.99×10^{-5}
1×10^{-5}	3.98×10^{-5}	1×10^{-4}	2.72×10^{-4}	1×10^{-5}	3.98×10^{-5}
5×10^{-5}	1.99×10^{-4}	1×10^{-3}	2.72×10^{-3}	5×10^{-5}	1.99×10^{-4}
1×10^{-4}	3.98×10^{-4}	-	-	1×10^{-4}	3.98×10^{-4}

Table 6. Absorbed radiation dose (Gy) and dilutions in buffer after γ-irradiation of *E. coli* bacteria for various media in the study of the survival curves.

wsE2 + HBC			wsP4 + HBC			T and VitC		
Dose	Air / N$_2$O	Argon	Dose	Air / N$_2$O	Argon	Dose	Air / N$_2$O	Argon
0, 30	2µl/250ml	2µl/250ml	0	2µl/250ml	2µl/250ml	0	2µl/200ml	2µl/200ml
90	2µl/200ml	2µl/200ml	50	2µl/250ml	2µl/250ml	50	3µl/200ml	2µl/200ml
150	3µl/100ml	2µl/100ml	120	2µl/200ml	2µl/200ml	120	2µl/100ml	2µl/100ml
220	4µl/100ml	3µl/100ml	250	4µl/100ml	3µl/100ml	200	3µl/100ml	2µl/100ml
300	4µl/100ml	4µl/100ml	400	3µl/50ml	3µl/100ml	300	3µl/50ml	2µl/50ml
450	3µl/50ml	3µl/50ml	600	4µl/50ml	3µl/50ml	450	4µl/50ml	3µl/50ml
600	4µl/50ml	4µl/50ml						

Procedure:

- take samples of 10 ml of the batch culture ($OD_{580} = 0.4$)

- harvest the bacteria by centrifugation (10 min, 4000 rpm, 25°C)

- wash with buffer, vortex well and centrifuge (10 min, 4000 rpm, 25°C)

- resuspend pellets in 10 ml buffer as well as in 10 ml buffer with different concentrations of substances (*e.g.* hormones, vitamins)

- incubate for 1 hour at room temperature

- dilute the samples by adding 3 µl of each sample to 200 ml buffer

- spread 100 µl of each sample onto 3 Petri dishes

- incubate o/n at 37°C

- count the surviving colonies formed

- calculate the N/N_0 ratio, whereby N_0 is the number of untreated colonies and N the number of colonies after treatment

- draw a diagram of the N/N_0 ratio versus the concentration of the tested substrate

5.8.4.3. *Survival curves under γ-irradiation*

Procedure:

- take samples of 10 ml of the batch culture ($OD_{580} = 0.4$)

- harvest the bacteria by centrifugation (10 min, 4000 rpm, 25°C)

- wash with buffer, vortex well and centrifuge (10 min, 4000 rpm, 25°C)

- resuspend pellets in 10 ml buffer as well as in 10 ml buffer with different substances and mixtures of substances ($1x10^{-5}$ mol.L^{-1} wsE2, $3.98x10^{-5}$ mol.L^{-1} HBC; $1x10^{-4}$ mol.L^{-1} wsP4, $2.72x10^{-4}$ mol.L^{-1} HBC, $1x10^{-4}$ mol.L^{-1} wsE2 + $1x10^{-4}$ mol.L^{-1} wsP4; $5x10^{-5}$ mol.L^{-1} T (0.5% ethanol), $5x10^{-5}$ mol.L^{-1} vitC (0.5% ethanol), $5x10^{-5}$ mol.L^{-1} T + $5x10^{-5}$ mol.L^{-1} vitC (0.5% ethanol))

- incubate the samples for 1h at room temperature

- saturate the aliquot-parts of the samples with air, argon or N_2O

- irradiate with γ-rays at various doses

- dilute with buffer according to Table 6

- spread 100 µl of each sample onto 3 Petri dishes

- incubate o/n at 37°C

- count the surviving colonies formed

- calculate the N/N_0 ratio, whereby N_0 is the number of untreated colonies and N the number of colonies after treatment
- draw a diagram of the N/N_0 ratio versus the absorbed radiation dose (Gy)
- calculate ΔD_{37} values from each survival curve by subtracting the D_{37} buffer value from each individual D_{37} value

6. RESULTS

6.1. Actinometry

The intensity of the emitted light of the UV-lamp in the irradiation vessel (Figure 15) was determined by monochloracetic acid actinometer (93,94). Figures 19 and 20 depict the established Cl⁻ calibration line and the illumination curve, respectively. According to eq. 46, I_0 was calculated. The dilution factor of 50 was also considered in the calculations.

$$I_0 = (2.2 \times 10^{-5} / 2) \times (6.023 \times 10^{23} / 0.36 \times 10^3) \times 50 = 0.92 \times 10^{18} \text{ hv.ml}^{-1}.\text{min}^{-1} \quad (48)$$
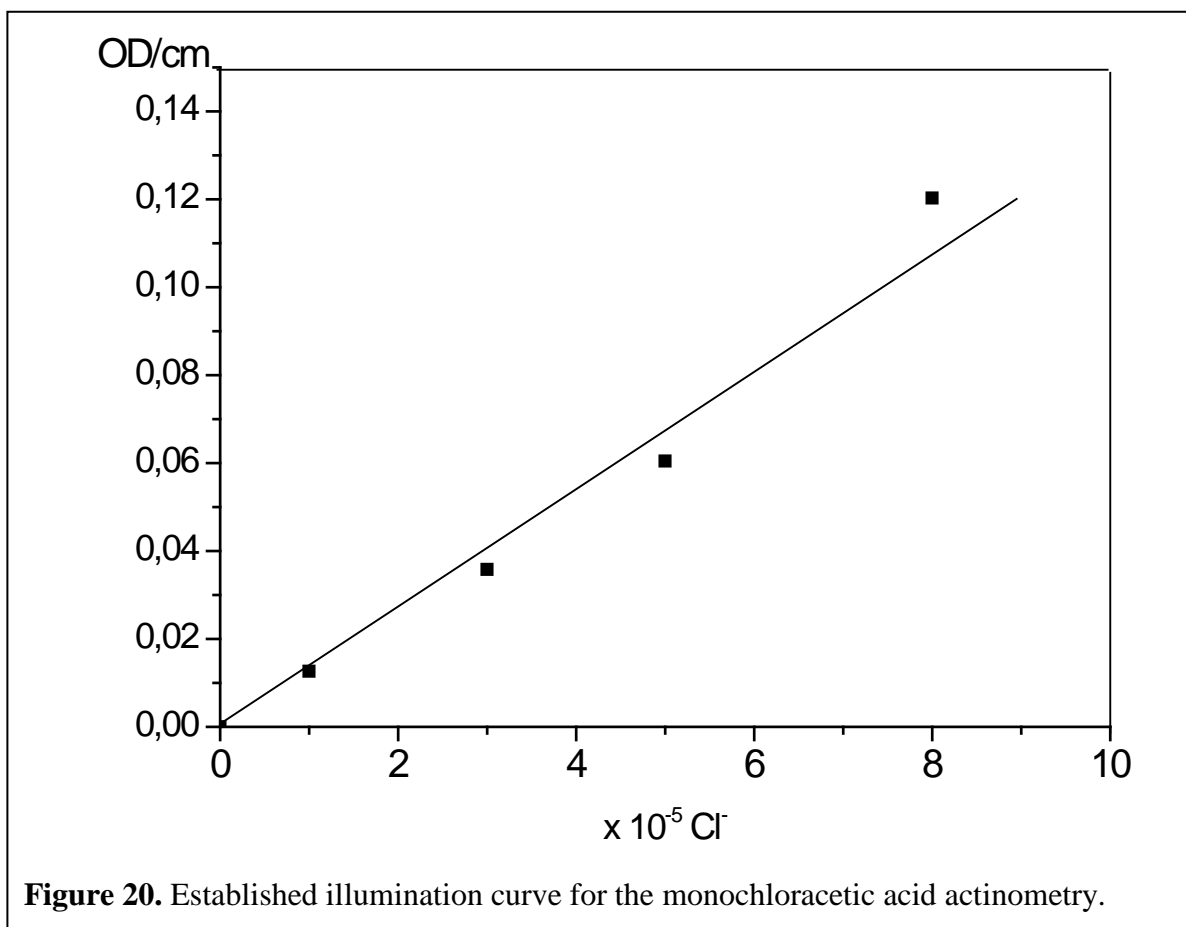
6.2. Electron emission of hormones

Due to previous studies with hormones (47) and other organic compounds (*e.g.* 51), which proved their ability to emit electrons, it was of interest to study further hormones in this respect. As the ejection of electrons is dependent on a numbers of factors such as temperature and pH, besides molecular structure and substituents, these aspects were investigated, as well.

6.2.1. Phytohormone GEN

GEN is a molecule with estrogenic properties and similar structure to E2. The latter has already been proven to emit electrons from its excited singlet state in water-ethanol solvent (47). Thus, it appeared of interest to investigate the yield of ejected electrons of GEN, too, in order to compare it with those of E2.

As already published (112), GEN was tested in a concentration range from 2.5×10^{-5} up to 1×10^{-4} mol/L at pH ~7.4 as a function of the absorbed UV quanta. Results of concentrations lower than 1×10^{-5} mol/L were not well reproducible, because of the weak absorption of GEN at 254 nm. Because of its high solubility in water, the experiments were conducted in air-free, aqueous solutions, using 1×10^{-2} chloroethanol as scavenger for e^-_{aq}. The mean values of several experiments are presented in Figure 21. Obviously, the yield of solvated electrons passes a maximum after absorption of ~3.5×10^{19} quanta. ml⁻¹, independent from the specific substrate concentration. Only for a short period after the start of the irradiation procedure, the yield of e^-_{aq} shows a linear correlation with the absorbed UV quanta. Therefore, the initial quantum yield, $Q(e^-_{aq})$, of each concentration

Figure 19. Established Cl⁻ calibration line for the monochloracetic acid actinometry.

Figure 20. Established illumination curve for the monochloracetic acid actinometry.
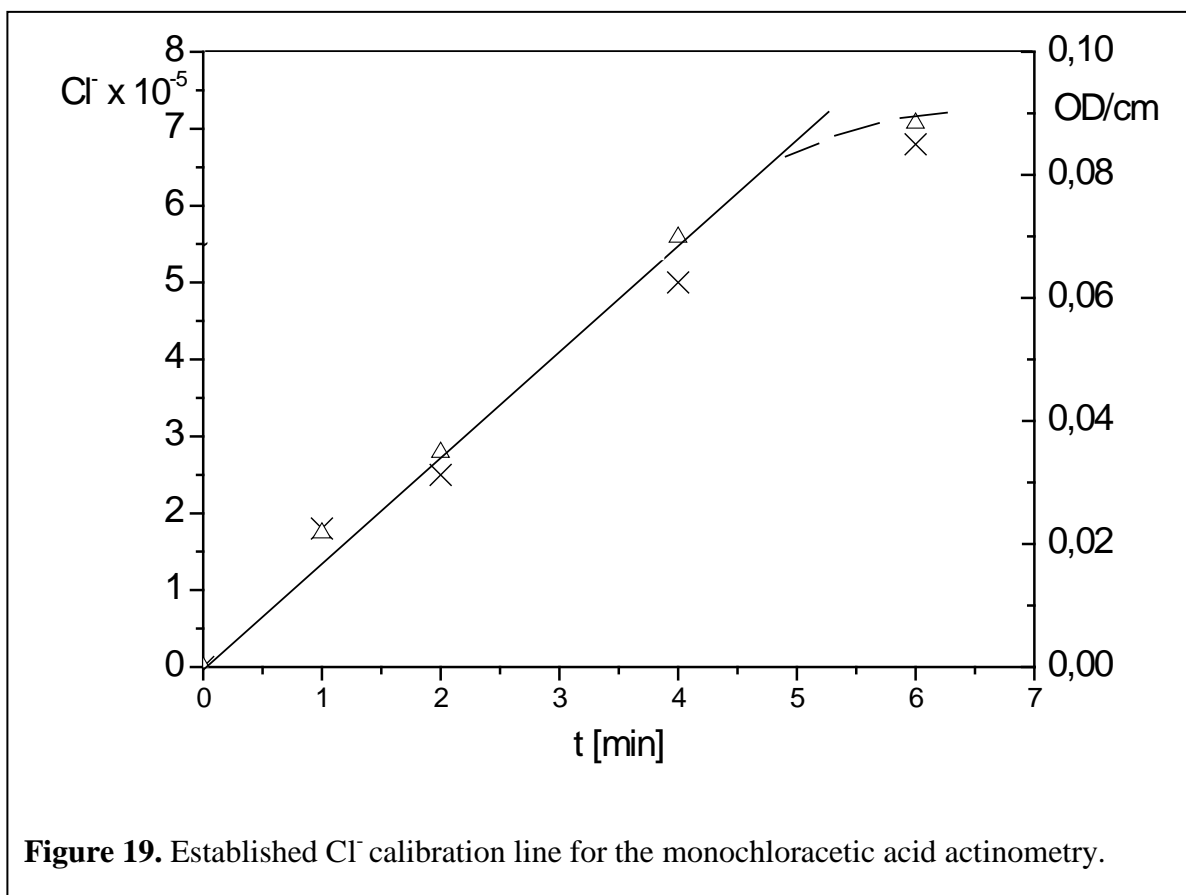
Figure 21. Electron emission (e⁻$_{aq}$, mol/L) from A: 2.5×10^{-5} mol/L GEN, B: 5×10^{-5} mol/L GEN, C: 7.5×10^{-5} mol/L GEN and D: 1×10^{-4} mol/L GEN in air-free, aqueous solution (pH~7.4, 37°C) as a function of the absorbed UV quanta (hv/ml; λ=254 nm). **Inset:** Initial Q(e⁻$_{aq}$) yields calculated from the linear part of the corresponding curves (112).
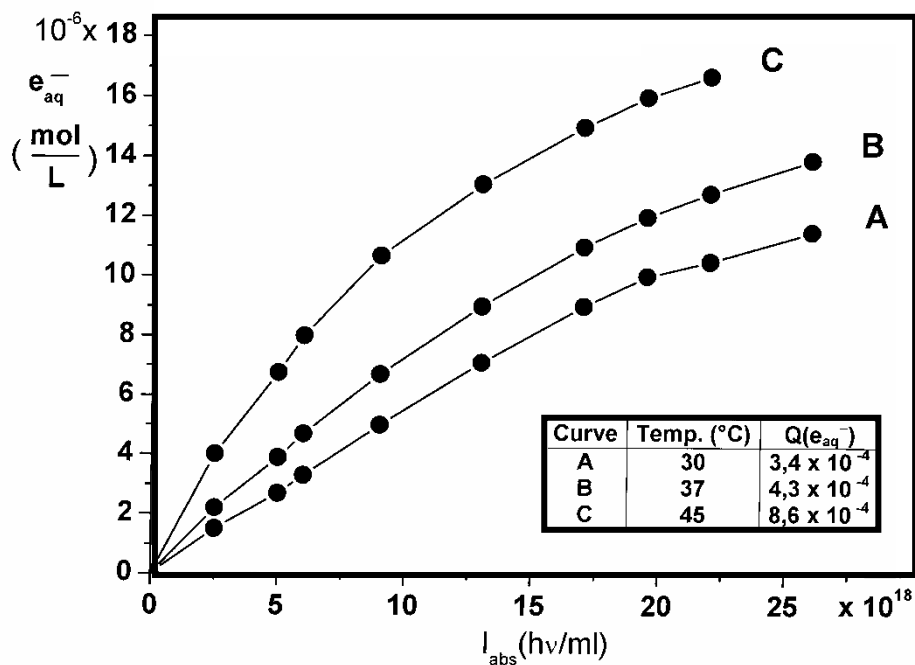
Figure 22. Electron emission (e⁻$_{aq}$, mol/L) from 5×10^{-5} mol/L GEN at a temperature of A: 30°C, B: 37°C and C: 45°C, in air-free, aqueous solution (pH ~7.4) as a function of the absorbed UV quanta (hv/ml; λ=254 nm). **Inset:** Initial Q(e⁻$_{aq}$) yields calculated from the linear part of the corresponding curves (112).

was calculated from the starting linear part of the specific curve. The obtained values are given as inset in Figure 21, whereby with rising substrate concentration the corresponding $Q(e^-_{aq})$ values are decreasing from $Q(e^-_{aq})=5.4\times10^{-4}$ for 2.5×10^{-5} mol/L GEN to $Q(e^-_{aq})=2.5\times10^{-4}$ for 1×10^{-4} mol/L GEN.

The effect of the temperature on the electron ejection of GEN was also investigated, as the temperature is known to influence the yield of the ejected electrons from excited substrate molecules essentially (52). In Figure 22 the yields of e^-_{aq} (mol/L) at 30 (curve A), 37 (curve B) and 45°C (curve C) are presented as a function of absorbed UV quanta. Again, only at low UV doses, the yield of e^-_{aq} shows a linear correlation with the absorbed UV quan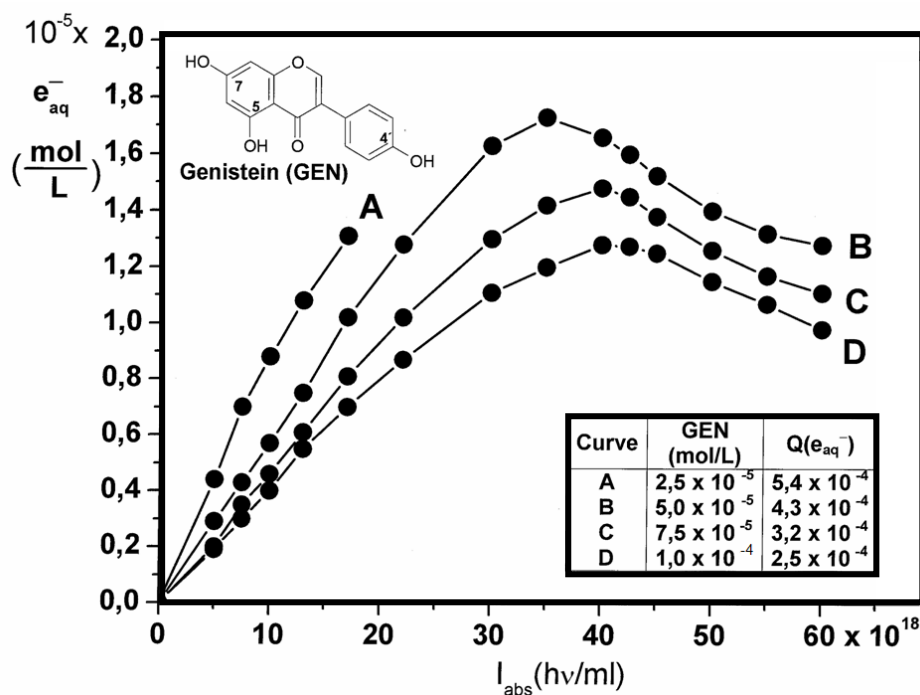ta. Thus, the initial quantum yields, $Q(e^-_{aq})$, were calculated (Figure 22, inset), which are strongly increasing with rising temperature. With increase of the absorbed UV quanta, each curve tends to a maximum.

Hormone (mol/L)	ε_{254} (L.mol^{-1}.cm^{-1})
A 1×10^{-2} ClEtOH	0
B 1×10^{-5} P4	21000
C 5×10^{-5} P4	13500
D 1×10^{-5} T	18000
E 5×10^{-5} T	14000

Figure 23. Absorption spectra of A: 1×10^{-2} mol/L chloroethanol, B: 1×10^{-5} mol/L P4, C: 5×10^{-5} mol/L P4, D: 1×10^{-5} mol/L T and E: 5×10^{-5} mol/L T in a solvent-mixture of 40% triply-distilled water and 60% p.a. ethanol at 37°C. **Inset:** Calculated molar extinction coefficients, ε_{254}, of the substrates (98).
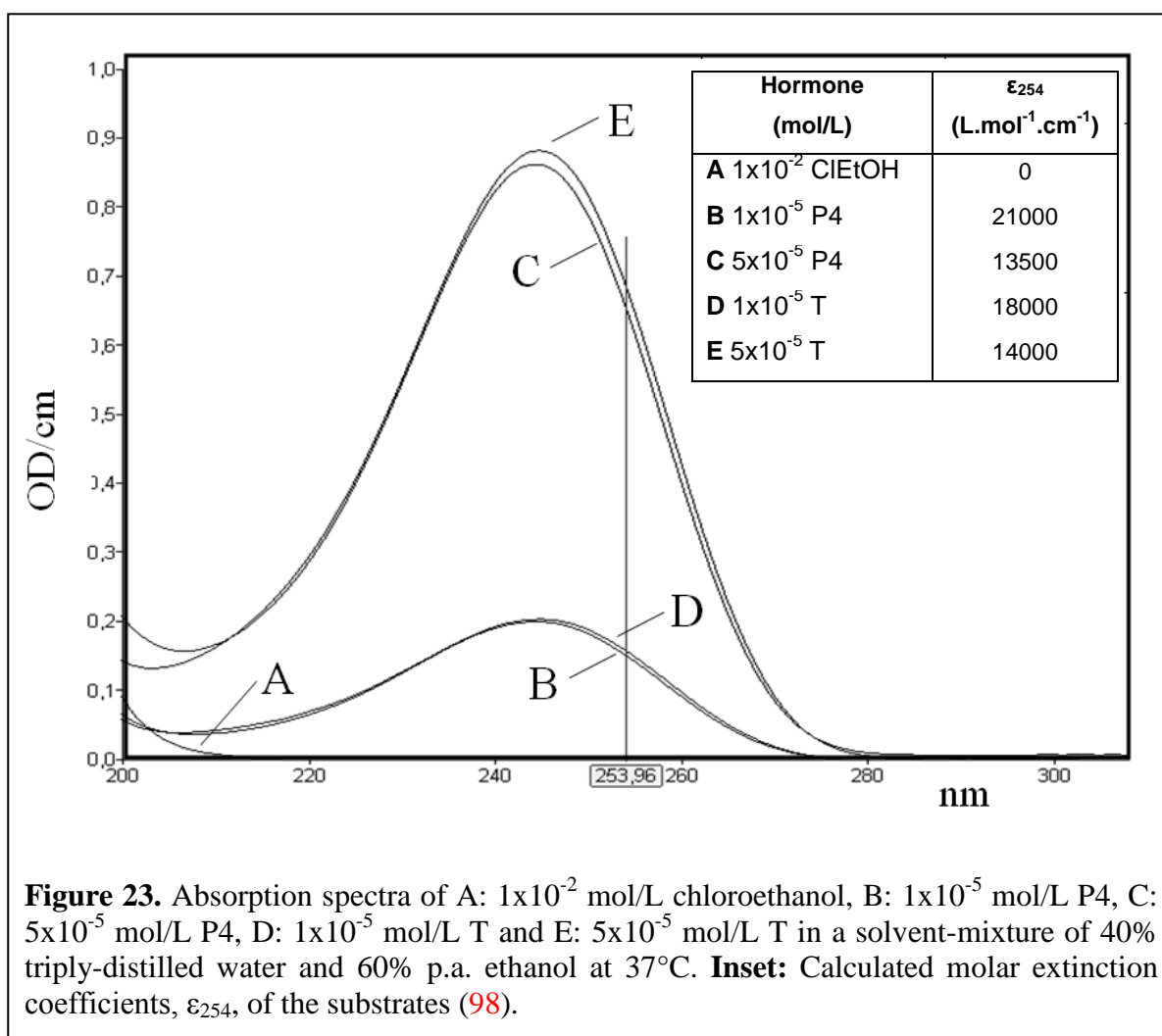
6.2.2. T alone and in mixture with P4

After the finding, that the female sex hormones E2 and P4 in water-ethanol solvent are both able to emit electrons when UV-excited in their singlet state (47), the question arose, if the same is true for the male sex hormone T, as well.

As already published (98), first, the absorption spectra of chloroethanol, T and P4 in several concentrations, dissolved in 40% triply-distilled water and 60% p.a. ethanol, were measured. The results are presented in Figure 23. It can be seen, that $1x10^{-2}$ mol/L chloroethanol, which was used as electron scavenger, does not absorb at λ=254 nm, which is the wavelength of the used UV-lamp. Therefore it can be stated, that the measured ejected electrons are exclusively emitted from the specific, studied hormone, and not from chloroethanol itself.

With the help of eq. 49 (Lambert-Beer-Law), the corresponding molar extinction coefficients (ε) at λ=254 nm were calculated (Figure 23, inset). The ε value of a compound is constant, regardless of the concentration. Here, the molar extinction coefficients were found to be decreasing with increasing substrate concentration.

$$\textbf{OD} = \textbf{ε x c x d} \tag{49}$$

OD…..… optical density
ε……...... molar absorption coefficient
c……….. concentration
d……….. optical pathway

The electron emission of T alone and in mixture with P4 was investigated in an air-free solvent mixture of 40% water and 60% ethanol (pH ~7.4) at 37°C. Figure 24 illustrates the yield of e^-_{aq} from $5x10^{-5}$ mol/L T (curve A), $5x10^{-5}$ mol/L P4 (curve B) and from their mixture consisting of $2.5x10^{-5}$ mol/L T and $2.5x10^{-5}$ mol/L P4 (curve C) in dependence of the absorbed UV dose (hν/L). The electron emission process of all three substrates exhibits several maxima. Only at low UV doses, the obtained yields of solvated electrons are proportional to the absorbed UV quanta. The calculated $Q(e^-_{aq})$ values of all maxima are given as inset in Figure 24. They decrease rapidly with dose.

In Figure 25, the obtained results of the investigations concerning the effect of the temperature on the yield of electrons emitted from UV-excited T, which are already published by Getoff et al. (99), are presented. Therefore, series of experiments were performed with $1x10^{-5}$ mol/L T in water-ethanol solvent at two different temperatures:

Figure 24. Electron emission (e^-_{aq}, mol/L) from A: 5×10^{-5} mol/L T, B: 5×10^{-5} mol/L P4 and C: mixture of 2.5×10^{-5} mol/L T and P4 each, in air-free water-ethanol solution (pH~7.4, 37°C) as a function of the absorbed UV quanta (hν/L; λ=254 nm).
Inset: $Q(e^-_{aq})$ values of the maxima (98).
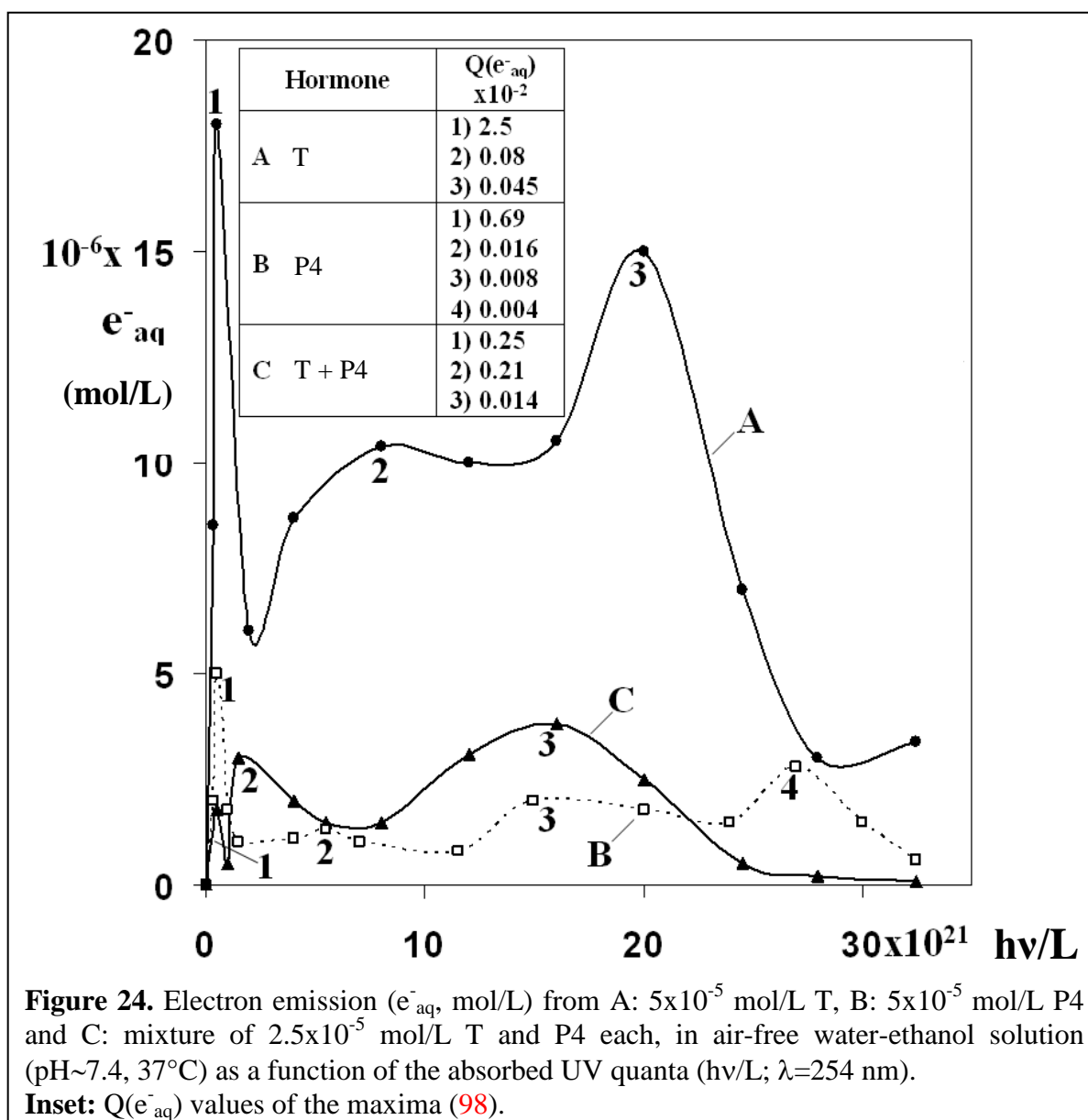
30°C (curve A) and 37°C (curve B). Both curves pass a sharp maximum at an absorbed UV dose of 8×10^{21} hν/L. However, it is clear, that with increasing temperature the yield of emitted e^-_{aq} from T also rises.

Due to the fact, that T and P4 are sensitive towards O_2, electron emission experiments were performed, whereby the hormones were either dissolved in aerated solvent (40% water and 60% ethanol; procedure A) and subsequently saturated with argon, or in an air-free solvent mixture, which was saturated with argon for 20 minutes prior to the dissolving of the hormone (procedure B). The obtained mean $Q(e^-_{aq})$ values using 5×10^{-5} mol/L hormone differ by two orders of magnitude, depending on the applied procedure (Table 7): When dissolving is done in aerated medium, the obtained quantum yields are much lower (cf. (99)).
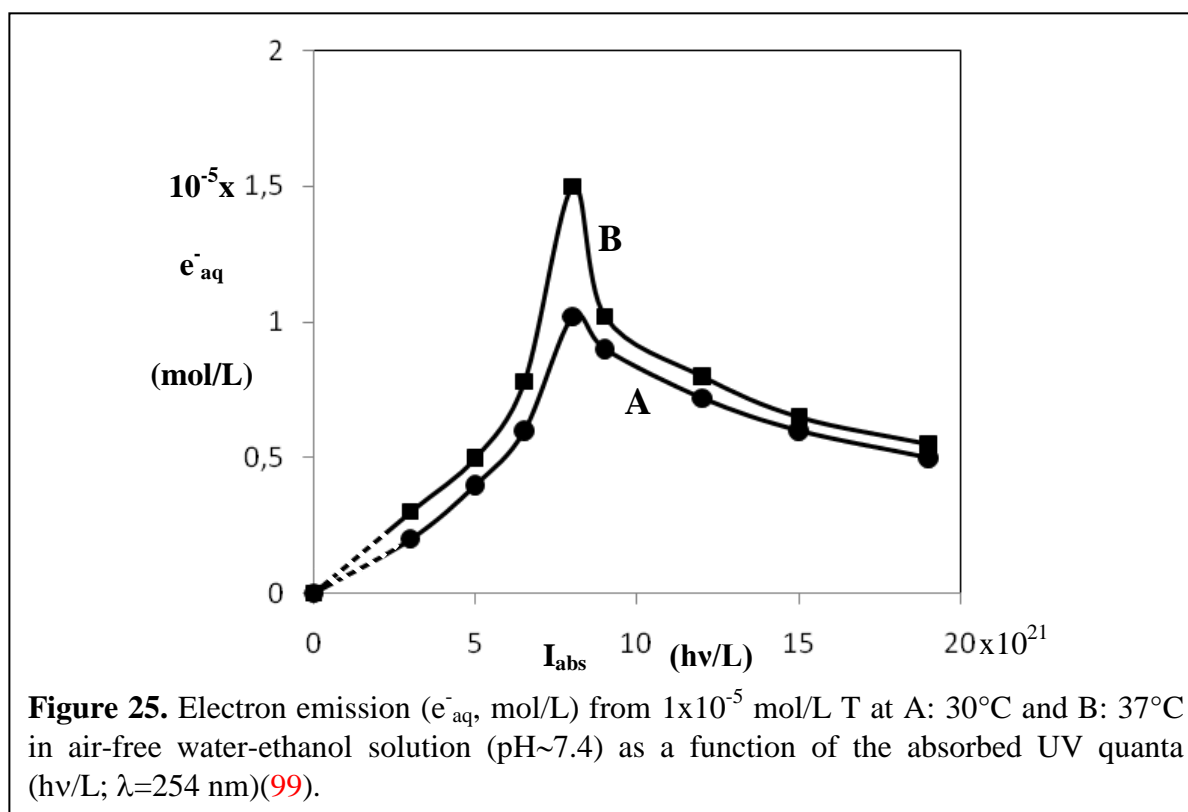
Figure 25. Electron emission (e^-_{aq}, mol/L) from 1×10^{-5} mol/L T at A: 30°C and B: 37°C in air-free water-ethanol solution (pH~7.4) as a function of the absorbed UV quanta (hv/L; λ=254 nm)(99).

Table 7. Oxygen sensitivity: Comparison of $Q(e^-_{aq})$ yields of T and P4 determined after dissolving in (A) aerated and (B) air-free mixture of 40% water and 60% ethanol at 37°C (98).

Hormone (mol/l)	$Q(e^-_{aq})$			
	TES		PRG	
	(A) aerated	(B) airfree	(A) aerated	(B) airfree
5×10^{-5}	4.65×10^{-4}	2.50×10^{-2}	4.00×10^{-4}	0.69×10^{-2}

6.2.3. Water-soluble E2, P4 and their mixture

For the experiments *in vitro* with *Escherichia coli* bacteria, the water-soluble hormones E2 and P4 (embedded in 2-hydroxypropyl-β-cyclodextrin, HBC) were applied, in order to be able to work in aqueous media without any addition of ethanol. Since both water-soluble hormones are complexed with HBC in different ratios (Table 3), not only wsE2 and wsP4, but also HBC alone had to be investigated in respect to its ability of electron ejection upon UV-excitation (already published (113)).

Figure 26 illustrates the yield of emitted e^-_{aq} from singlet excited 1×10^{-4} mol/L HBC as a function of the absorbed UV dose at 37°C. The obtained curve passes several maxima, whereby the yield of e^-_{aq} decreases slowly. Therefore, $Q(e^-_{aq})$ was calculated from each maximum and is given as inset (I) in Figure 26. It can be seen, that the $Q(e^-_{aq})$ values

Figure 26. Electron emission (e^-_{aq}, mol/L) from $1x10^{-4}$mol/L HBC in air-free, aqueous solution (pH ~7.4, 37°C) as a function of the absorbed UV quanta (hν/L; λ=254 nm). **Inset I**: $Q(e^-_{aq})$ values of the maxima. **Inset II**: The pH as a function of the absorbed UV dose (113).
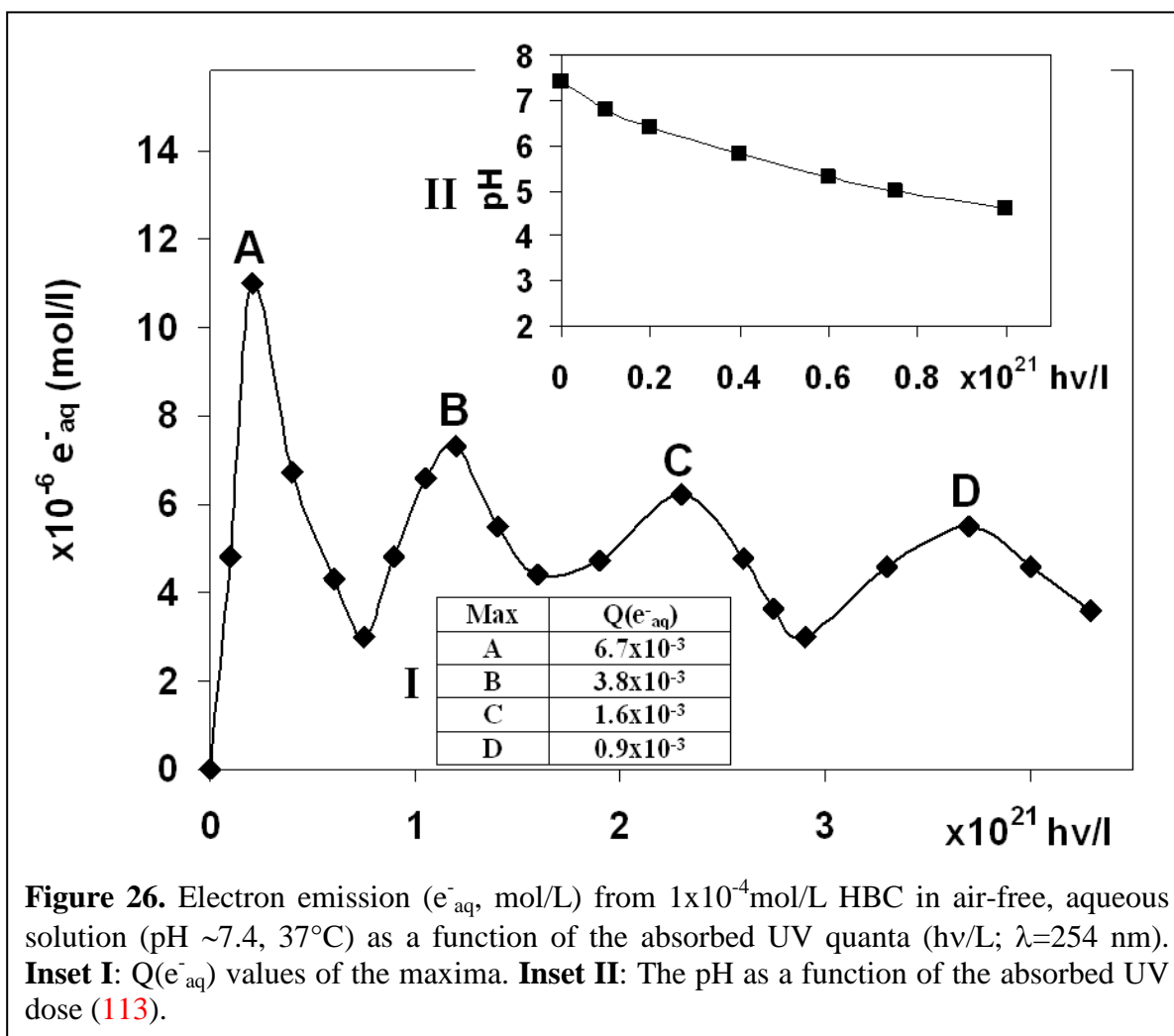
decrease with increasing UV dose. Simultaneously to the electron emission experiments, the pH was measured. The obtained results are presented in inset II of Figure 26. A strong correlation between rising absorbed UV dose and decreasing pH values was observed.

Subsequently, the same experiments were conducted with both water-soluble hormones. The obtained results for $1x10^{-4}$ mol/L wsE2 (in complex with $3.98x10^{-4}$ mol/L HBC) are depicted in Figure 27A. The yields of solvated electrons and the absorbed UV dose are proportional just at low irradiation doses. However, it can be seen that the obtained curve shows two maxima in the studied UV dose range. The calculated $Q(e^-_{aq})$ values from the maxima decrease with increasing absorbed UV dose (Figure 27A, inset). The curve obtained from $1x10^{-4}$ mol/L wsP4 (in complex with $2.72x10^{-4}$ mol/L HBC), passes three maxima (Figure 27B). However, it should be noted, that P4 was studied over a much larger UV dose in this respect. Interestingly, the yield of emitted e^-_{aq} is rising with increasing duration of UV-irradiation. The calculated $Q(e^-_{aq})$ values, which are presented
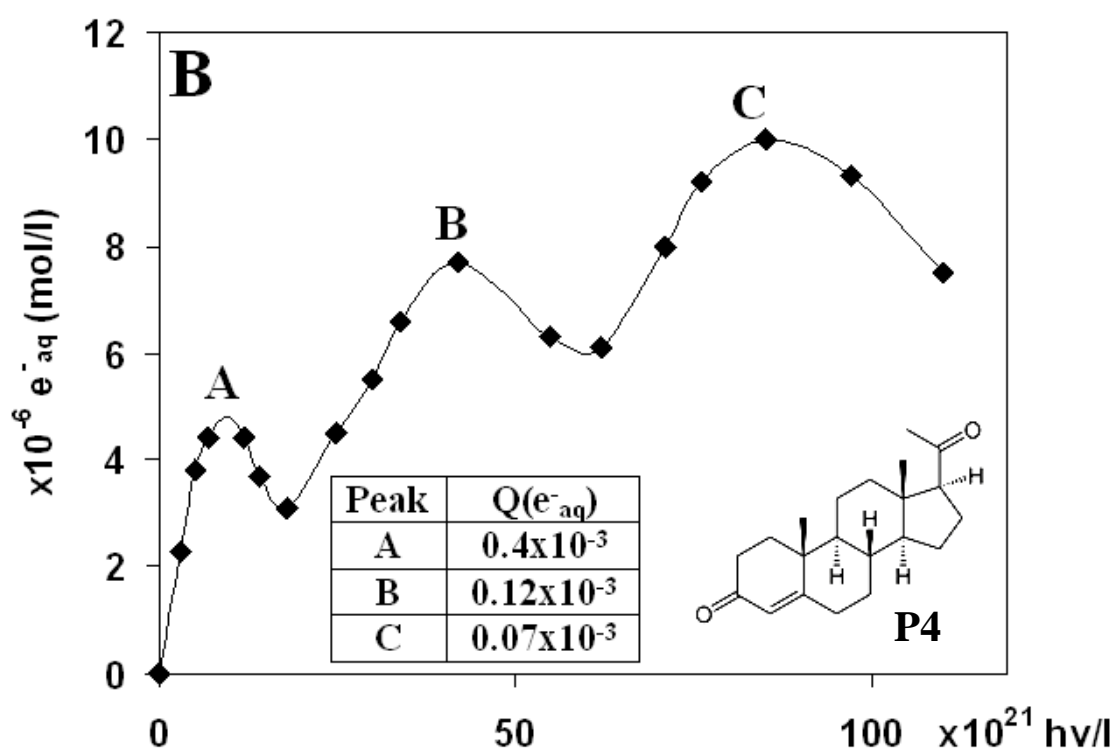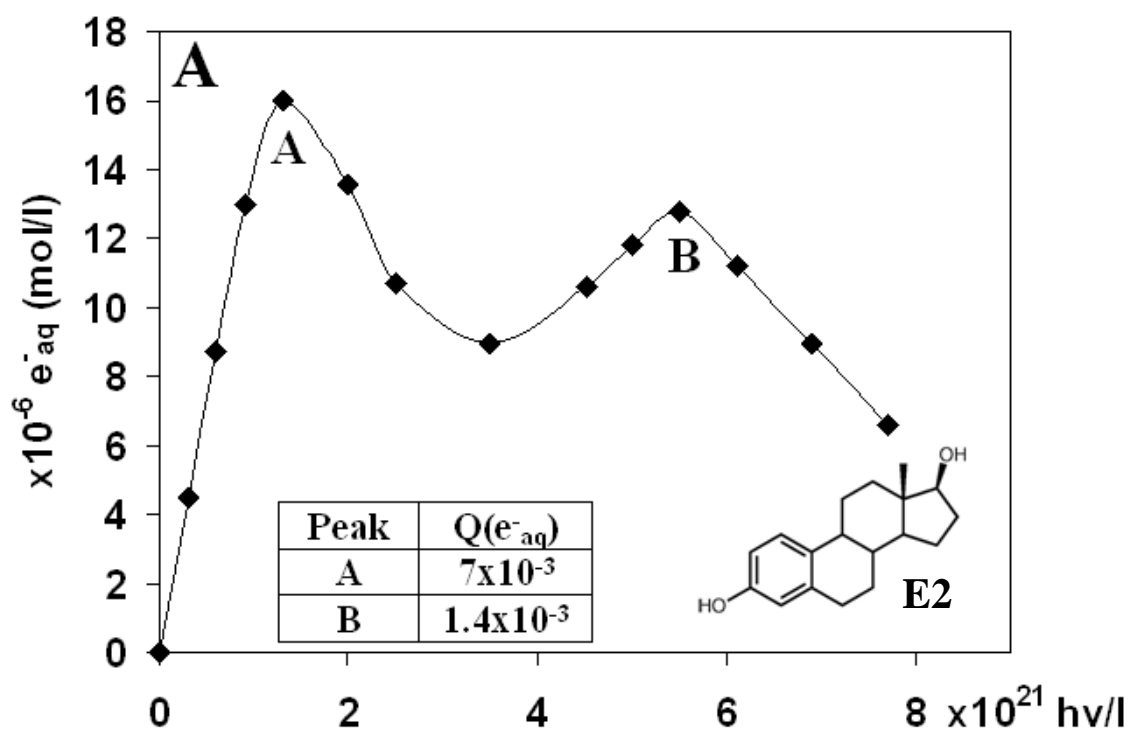
Figure 27. Electron emission (e$^-_{aq}$, mol/L) from A: 1×10^{-4} mol/L wsE2 (with 3.98×10^{-4} mol/L HBC) and B: 1×10^{-4} mol/L wsP4 (with 2.72×10^{-4} mol/L HBC) in air-free, aqueous solution (pH ~7.4, 37°C) as a function of the absorbed UV quanta (hv/L; λ=254 nm). **Insets**: Q(e$^-_{aq}$) values of the maxima. (113).
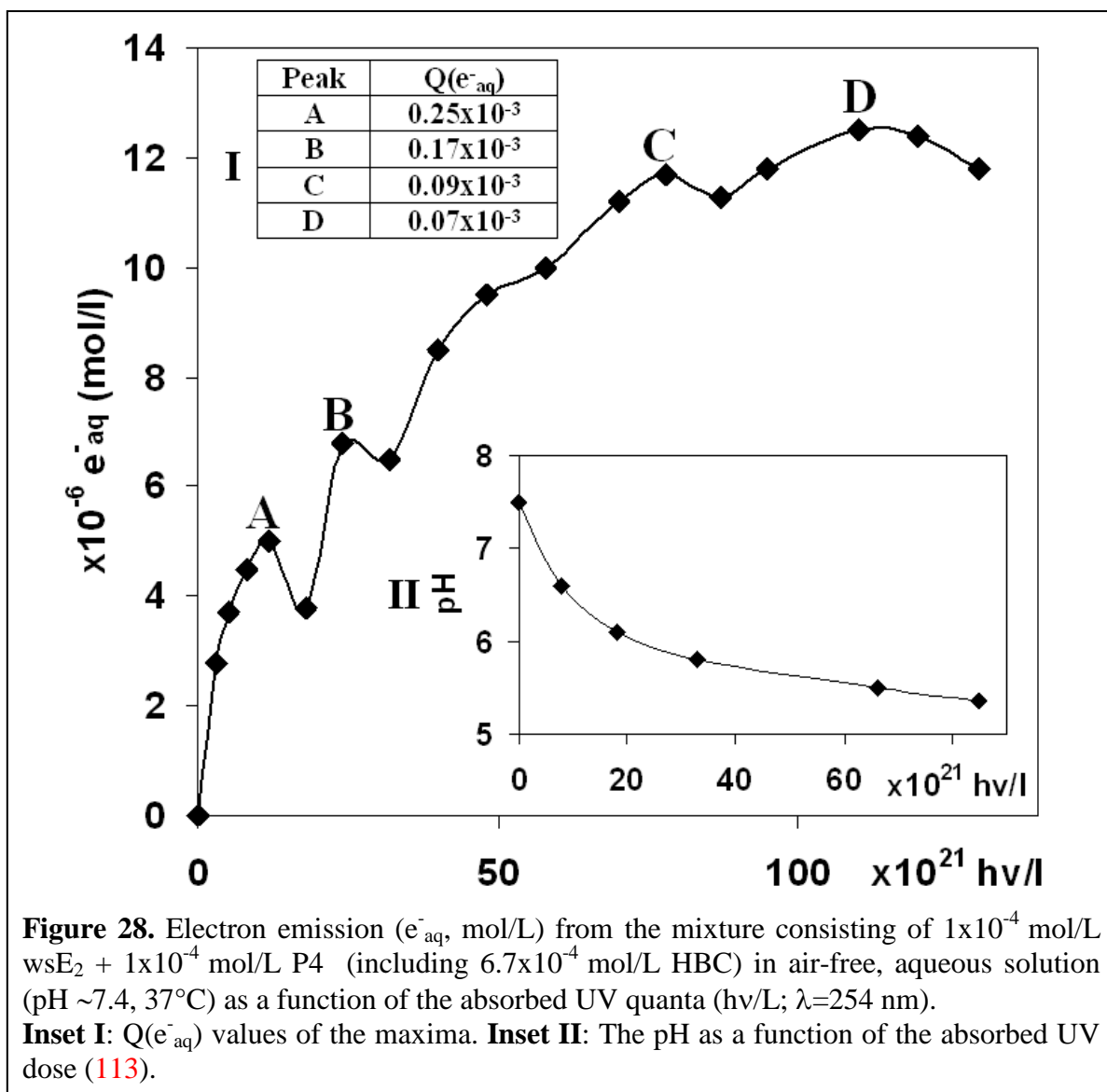
Figure 28. Electron emission (e^-_{aq}, mol/L) from the mixture consisting of 1×10^{-4} mol/L wsE$_2$ + 1×10^{-4} mol/L P4 (including 6.7×10^{-4} mol/L HBC) in air-free, aqueous solution (pH ~7.4, 37°C) as a function of the absorbed UV quanta (hv/L; λ=254 nm). **Inset I**: Q(e^-_{aq}) values of the maxima. **Inset II**: The pH as a function of the absorbed UV dose (113).

as inset in Figure 27B, are by an order of magnitude lower than those observed for wsE2 (Figure 27A, inset).

Finally, a combination of wsE2 and wsP4 was tested for its electron emission ability when irradiated with monochromatic UV-light (λ=254 nm), in order to examine possible mutual interactions of both hormones. The used concentrations were 1×10^{-4} mol/L wsE2 and 1×10^{-4} mol/L wsP4. Correspondingly, 6.7×10^{-4} mol/L HBC were also contained in the samples. The observed yields of solvated electrons, which result from all three substrates, are depicted in Figure 28 in dependence of the absorbed UV dose. It is obvious, that the yields increase with increasing absorbed UV dose by passing several small maxima. Simultaneously, the pH values decrease (Figure 28, inset II). Anyway, compared with Q(e^-_{aq}) from wsE2 or wsP4 alone, the calculated Q(e^-_{aq}) values from the mixture (Figure 28, inset I) are lower.

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6.3. HPLC-analyses

In order study the degradation of hormones and the formation of products under UV-irradiation, HPLC-analyses were performed. Furthermore, a possible regeneration of hormones in combination experiments with vitC, a very potent electron donor, was investigated by this method. Therefore, hormones or mixtures of hormones in an air-free solvent mixture of 40% triply-distilled water and 60% p.a. ethanol were UV-irradiated with monochromatic light (λ=254 nm). The temperature was kept constant at 37°C throughout the irradiation procedure. Finally, samples taken at various times of irradiation were analysed by HPLC.

6.3.1. T, P4 and their mixture

In Figures 29-31, the results of the HPLC-analyses of 5×10^{-5} mol/L T, 5×10^{-5} mol/L P4 and a mixture of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4, respectively, are presented. For all three substrates, eluent A (Table 4) was used.

T was found to have a retention peak of ~10,3 minutes under the applied HPLC-settings (Figure 29). It's relatively quickly degraded. While the unirradiated peak (Figure 29A) has an area of 2310 mAU*s, it is reduced to 121 mAU*s after an absorbed dose of 7.6×10^{20} hv/L (Figure 29C). After an absorption of 1.9×10^{21} hv/L, T couldn't be detected at all (Figure 29E). Simultaneously, the formation of a degradation product was observed, but with very low yields. But again, after an absorption of 1.9×10^{21} hv/L, it couldn't be detected any more.

Figure 30 presents the HPLC-chromatograms of 5×10^{-5} mol/L P4 UV-irradiated at various doses. Again, with increasing UV dose, the area of the peak (retention time: 15.8 min) decreases rapidly from 2584 mAU*s (Figure 30A), to 121 mAU*s after $I_{abs}=8\times10^{20}$ hv/L (Figure 30C), and to 0 mAU*s after 2×10^{21} absorbed quanta per liter (Figure 30F). Various small peaks from degradation products were found.

The HPLC-chromatograms of the UV-irradiated mixture of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4 are depicted in Figure 31. As the same eluent was used for T and P individually, the same retention times were observed in the mixture: 10.3 min for T and 15.8 min for P4. Both peaks of the mixture decrease rather fast with increasing absorbed UV dose. While only small and few product peaks were observed for T and P4 alone, the mixture of both leads to the formation of more degradation products with higher yields (Figure 31D).

Figure 29. HPLC-chromatograms of 5×10^{-5} mol/L T in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses ($\lambda = 254$ nm) at 37°C. A: non-irradiated T, B: T after $I_{abs} = 3.8 \times 10^{20}$ hv/L, C: T after $I_{abs} = 7.6 \times 10^{20}$ hv/L, D: T after $I_{abs} = 1.2 \times 10^{21}$ hv/L, E: T after $I_{abs} = 1.9 \times 10^{21}$ hv/L. For the HPLC analyses, eluent A (Table 4) was used.

Figure 30. HPLC-chromatograms of 5×10^{-5} mol/L P4 in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses (λ=254 nm) at 37°C. A: non-irradiated P4, B: P4 after I_{abs}=4×10^{20} hv/L, C: P4 after I_{abs}=8×10^{20} hv/L, D: P4 after I_{abs}=1.2×10^{21} hv/L, E: P4 after I_{abs}=2×10^{21} hv/L. For the HPLC analyses, eluent A (Table 4) was used.

Figure 31. HPLC-chromatograms of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4 in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses (λ=254 nm) at 37°C. A: non-irradiated mix, B: mix after I_{abs}=4.3×10^{20} hv/L, C: mix after I_{abs}=8.5×10^{20} hv/L, D: mix after I_{abs}=1.3×10^{21} hv/L, E: mix after I_{abs}=2.1×10^{21} hv/L. For the HPLC analyses, eluent A (Table 4) was used.

6.3.2. T, vitC and their mixture

It was postulated, that a potent electron donor like vitC could contribute to a possible regeneration of hormones, such as T, due to an electron transfer from the donor (vitC) to the acceptor (T). A remarkable reduction of the number of carcinogenic metabolites resulting from hormones after electron ejection could be achieved by regeneration processes. Thus, the degradation of $1x10^{-4}$ mol/L T, $1x10^{-4}$ mol/L vitC and their mixture ($1x10^{-4}$ mol/L T and C each) was studied at a physiological temperature of 37°C after various times of UV-irradiation. The results of the corresponding HPLC-analyses using eluent B (Table 4) are presented in Figures 32-34.

Due to the different eluent used (cf. Figure 29), the retention time of the testosterone peak was now observed at 21.7 minutes (Figure 32). The area of the peak degrades from 9080 mAU*s when not irradiated (Figure 32A) to 4060 mAU*s after an absorbtion of $4.7x10^{20}$ hv/L (Figure 32B), to 1084 mAU*s after $I_{abs}=9.4x10^{20}$ hv/L (Figure 32C) and to 22 mAU*s after $1.9x10^{21}$ absorbed quanta per liter (Figure 32D). At higher UV doses (Figure 32E,F), T couldn't be detected any more. It is interesting to note, that a formation of any degradation products was not observed at all.

The HPLC-chromatograms of UV-irradiated $1x10^{-4}$ mol/L vitC are depicted in Figure 33. With increasing absorbed UV dose, the area of the vitC peak (retention time: 4.3 min) decreases from 2034 mAU*s, when not irradiated (Figure 33A), to 1430 mAU*s after $I_{abs}=6.7x10^{20}$ hv/L (Figure 33C). Even after absorption of $3.4x10^{21}$ quanta per liter, a peak with an area of 74 mAU*s is found (Figure 33F).Again, no degradation products could be detected. Compared to T it can be stated, that the degradation of vitC is much slower, as it could still be detected after much higher absorbed UV quanta.

Figure 34 presents the HPLC-chromatograms of the UV-irradiated mixture of $1x10^{-4}$ mol/L T and $1x10^{-4}$ mol/L vitC at various doses. Testosterone degraded much slower when irradiated in mixture with vitC. The area of the observed peak degrades from 10360 mAU*s (Figure 34A) to 1834 mAU*s after absorption of $2x10^{21}$ hv/L (Figure 34D). At that UV dose, individually irradiated T was degraded to almost 100% (Figure 32D). In mixture, T was still detected at an absorbed dose of $5x10^{21}$ quanta per liter with an peak area of 74 mAU*s (Figure 34D). VitC shows a delayed degradation, as well. Starting with an area of 3730 mAU*s, when not irradiated (Figure 34A), the value decreases to 420 at $5x10^{21}$ hv/L (Figure 34F), which is a much higher yield at a much higher absorbed radiation dose as observed for individually irradiated vitC .
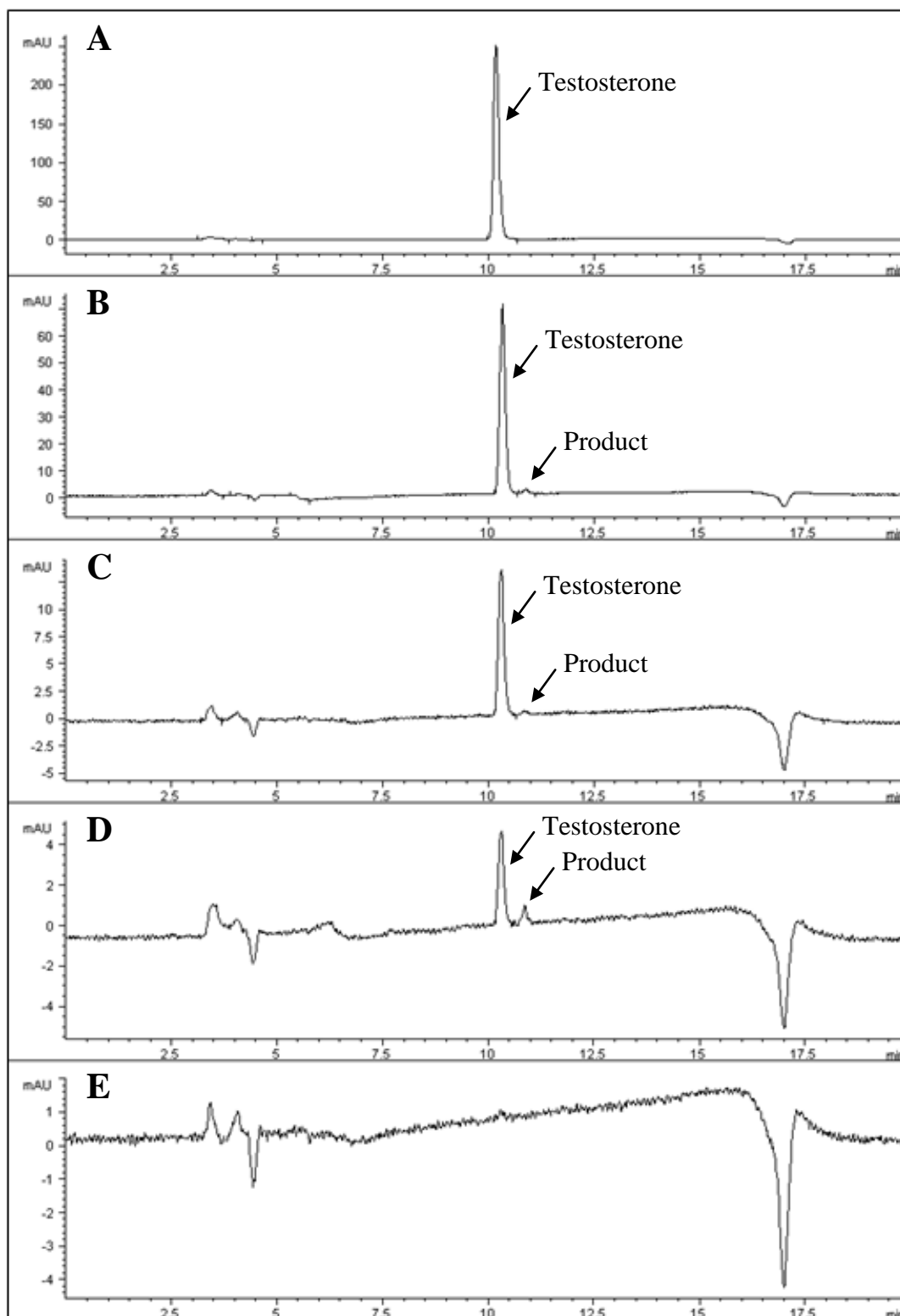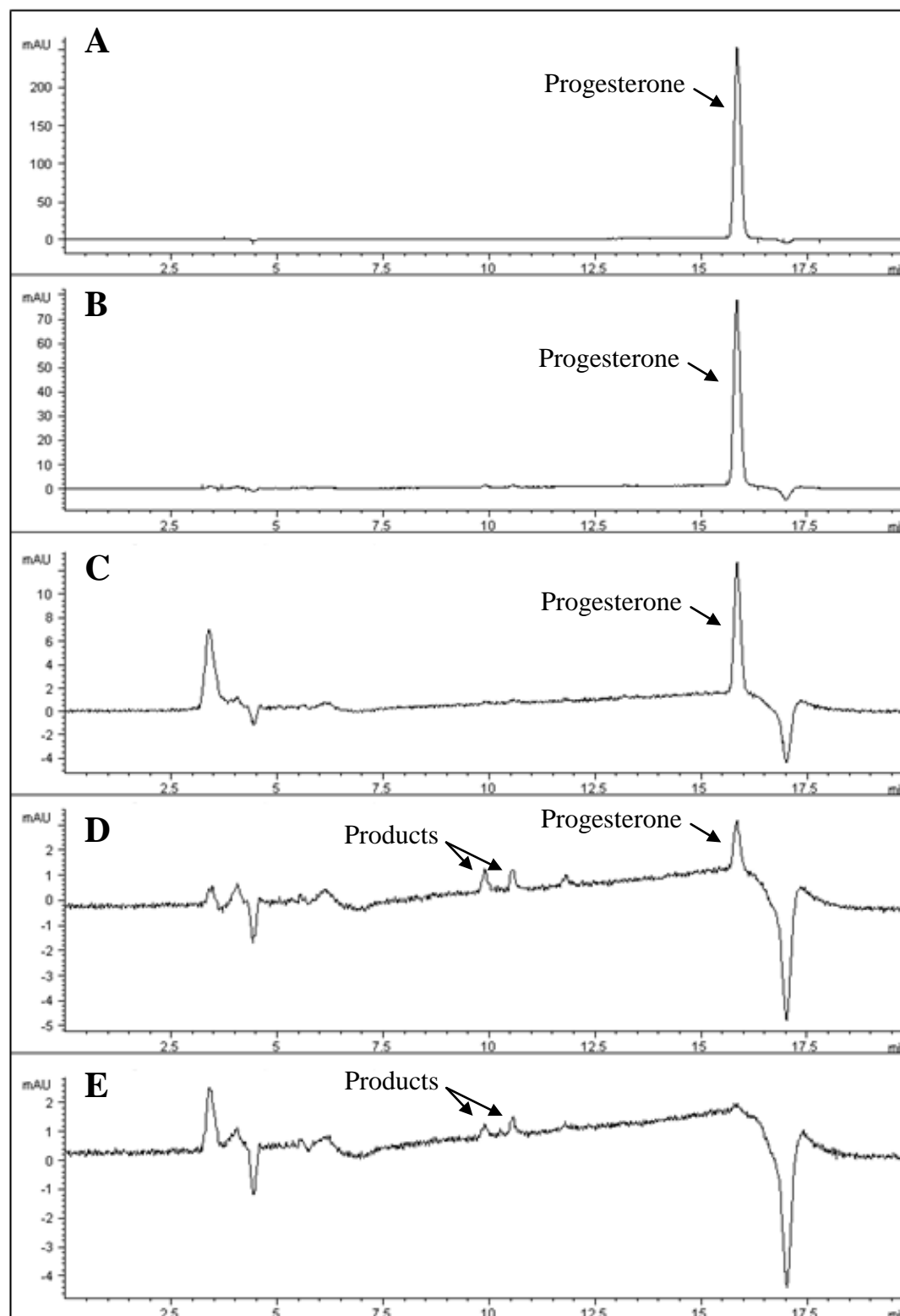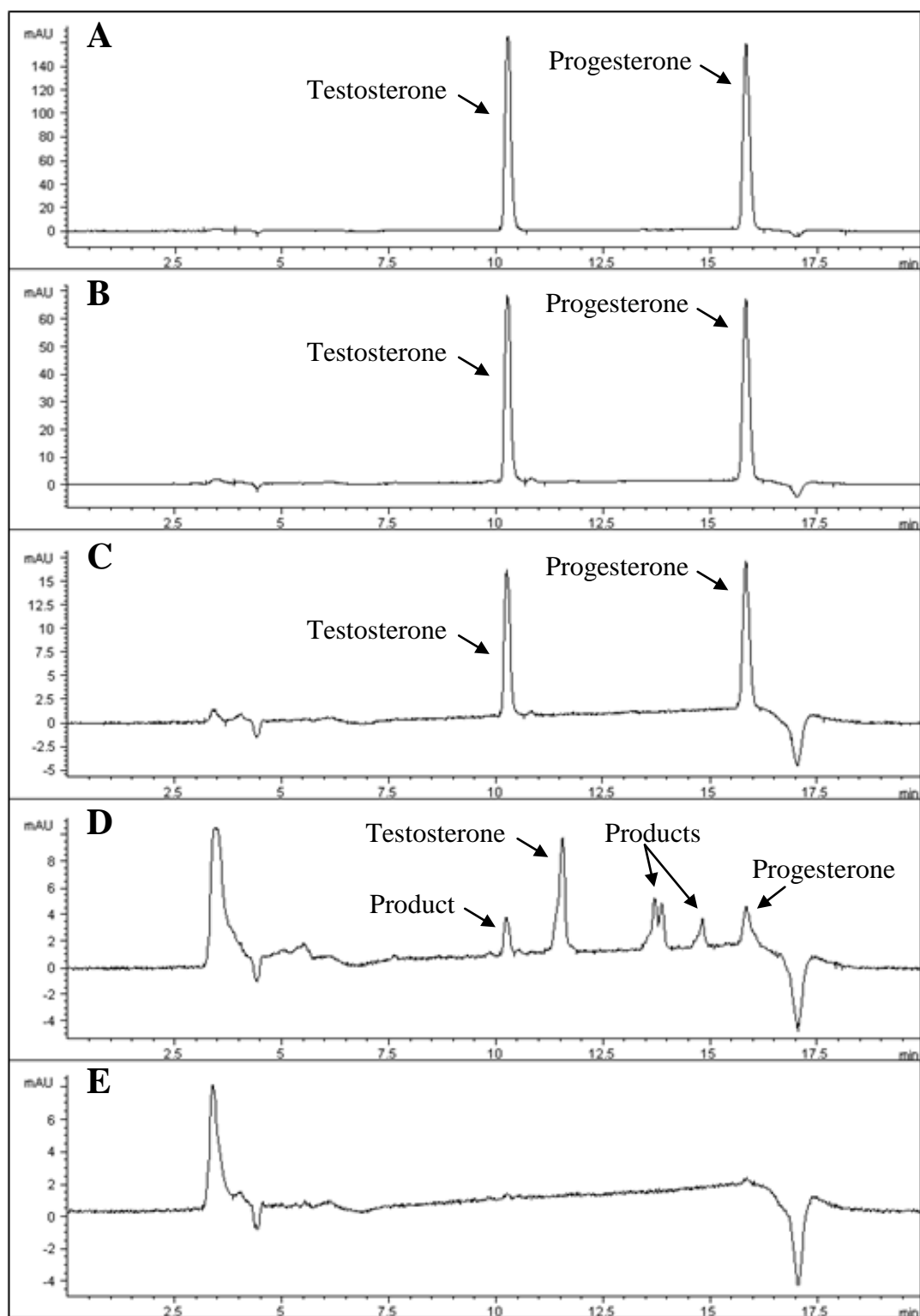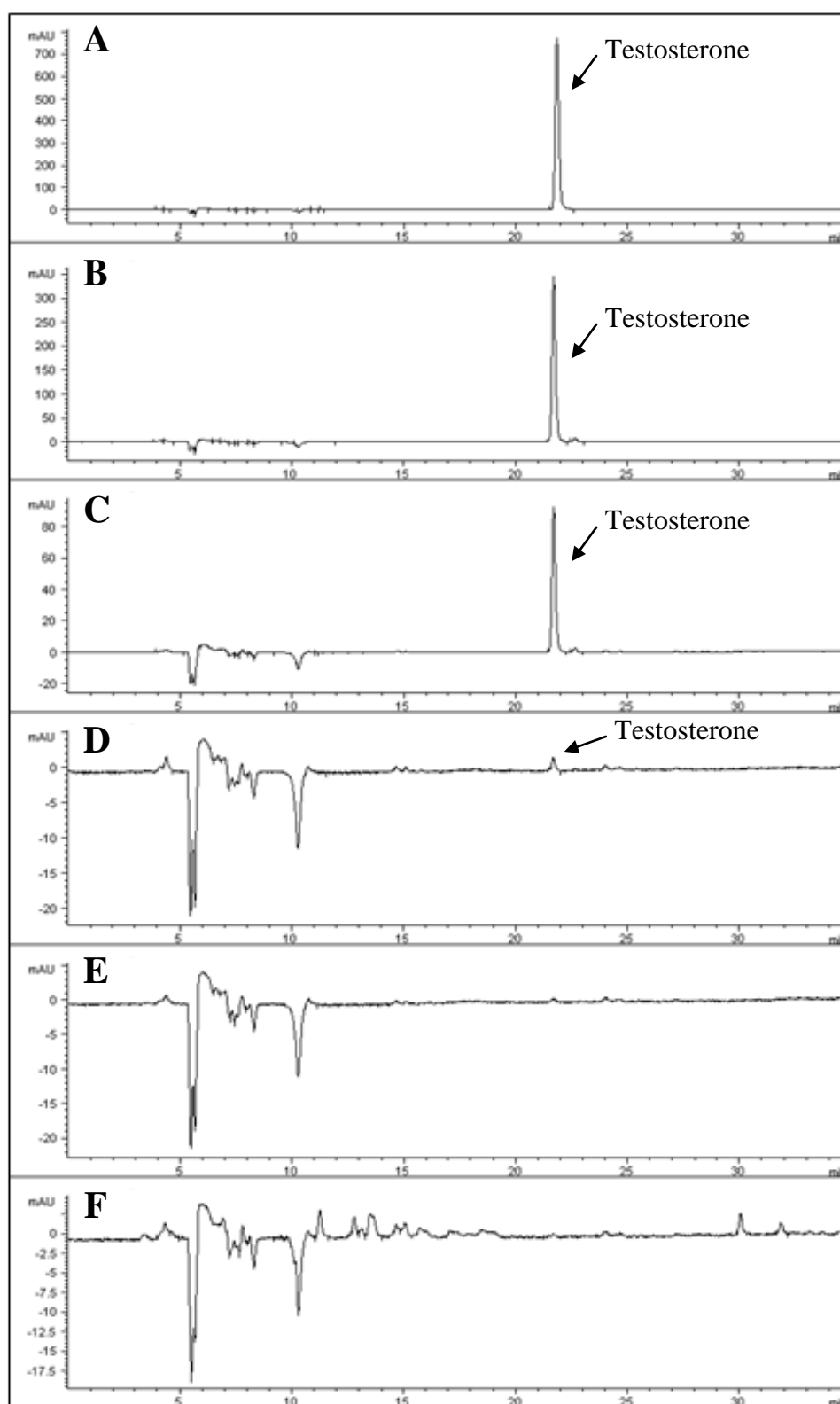
Figure 32. HPLC-chromatograms of 1×10^{-4} mol/L T in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses (λ=254 nm) at 37°C. A: non-irradiated T, B: T after I_{abs}=4.7x10^{20} hν/L, C: T after I_{abs}=9.4x10^{20} hν/L, D: T after I_{abs}=1.9x10^{21} hν/L, E: T after I_{abs}=2.8x10^{21} hν/L, F: T after I_{abs}=4.7x10^{21} hν/L. For the HPLC analyses, eluent B (Table 4) was used.
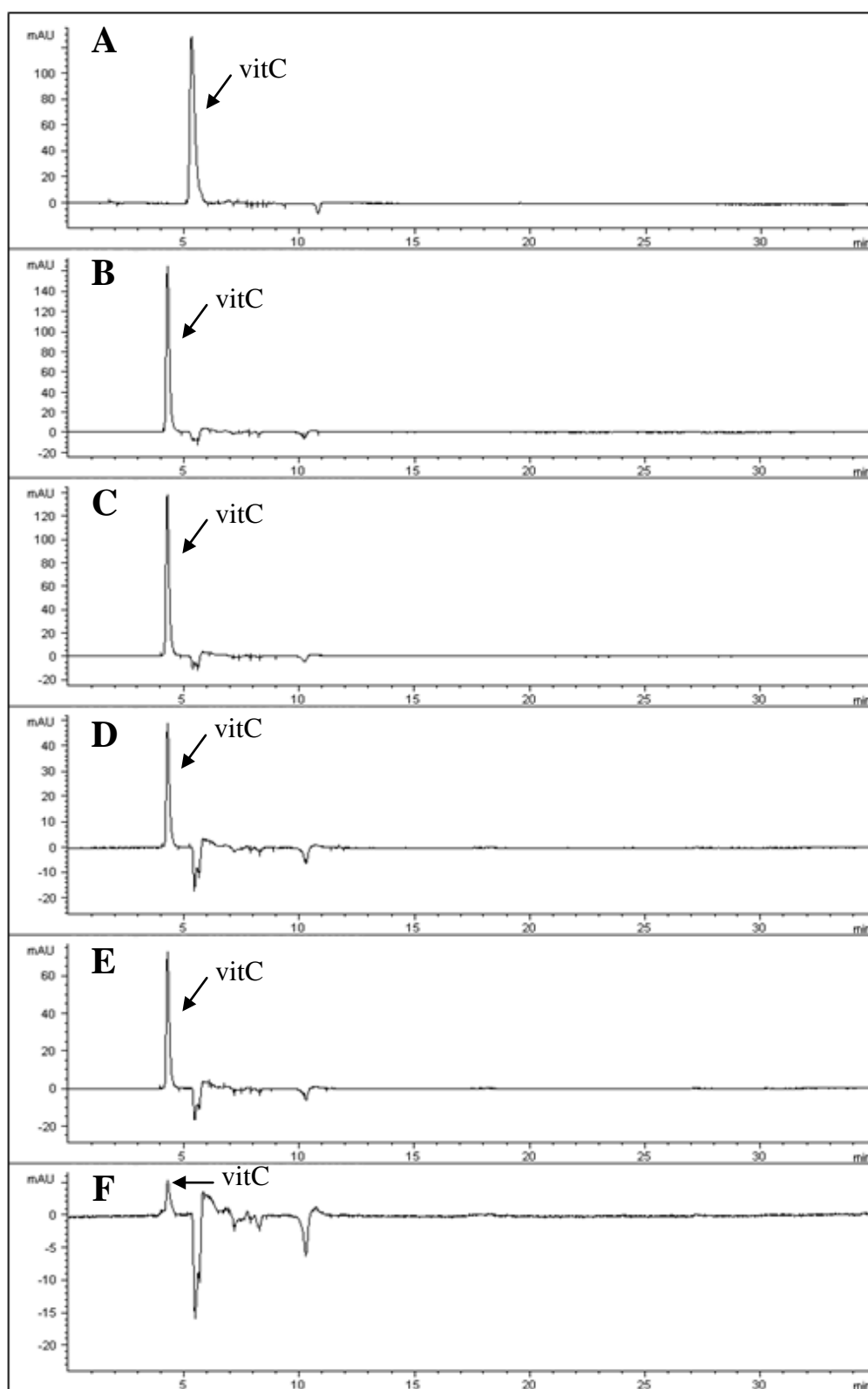
Figure 33. HPLC-chromatograms of 1×10^{-4} mol/L vitC in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses (λ=254 nm) at 37°C. A: non-irradiated vitC, B: vitC after $I_{abs}=3.4 \times 10^{20}$ hv/L, C: vitC after $I_{abs}=6.7 \times 10^{20}$ hv/L, D: vitC after $I_{abs}=1.3 \times 10^{21}$ hv/L, E: vitC after $I_{abs}=2 \times 10^{21}$ hv/L, F: vitC after $I_{abs}=3.4 \times 10^{21}$ hv/L. For the HPLC analyses, eluent B (Table 4) was used.

Figure 34. HPLC-chromatograms of 1×10^{-4} mol/L T and 1×10^{-4} mol/L vitC in water-ethanol solvent (pH ~7.4), UV-irradiated with various doses (λ=254 nm) at 37°C. A: non-irradiated mix, B: mix after I_{abs}=5×10^{20} hν/L, C: mix after I_{abs}=1×10^{21} hν/L, D: mix after I_{abs}=2×10^{21} hν/L, E: mix after I_{abs}=3×10^{21} hν/L. F: mix after I_{abs}=5×10^{21} hν/L. For the HPLC analyses, eluent B (Table 4) was used.

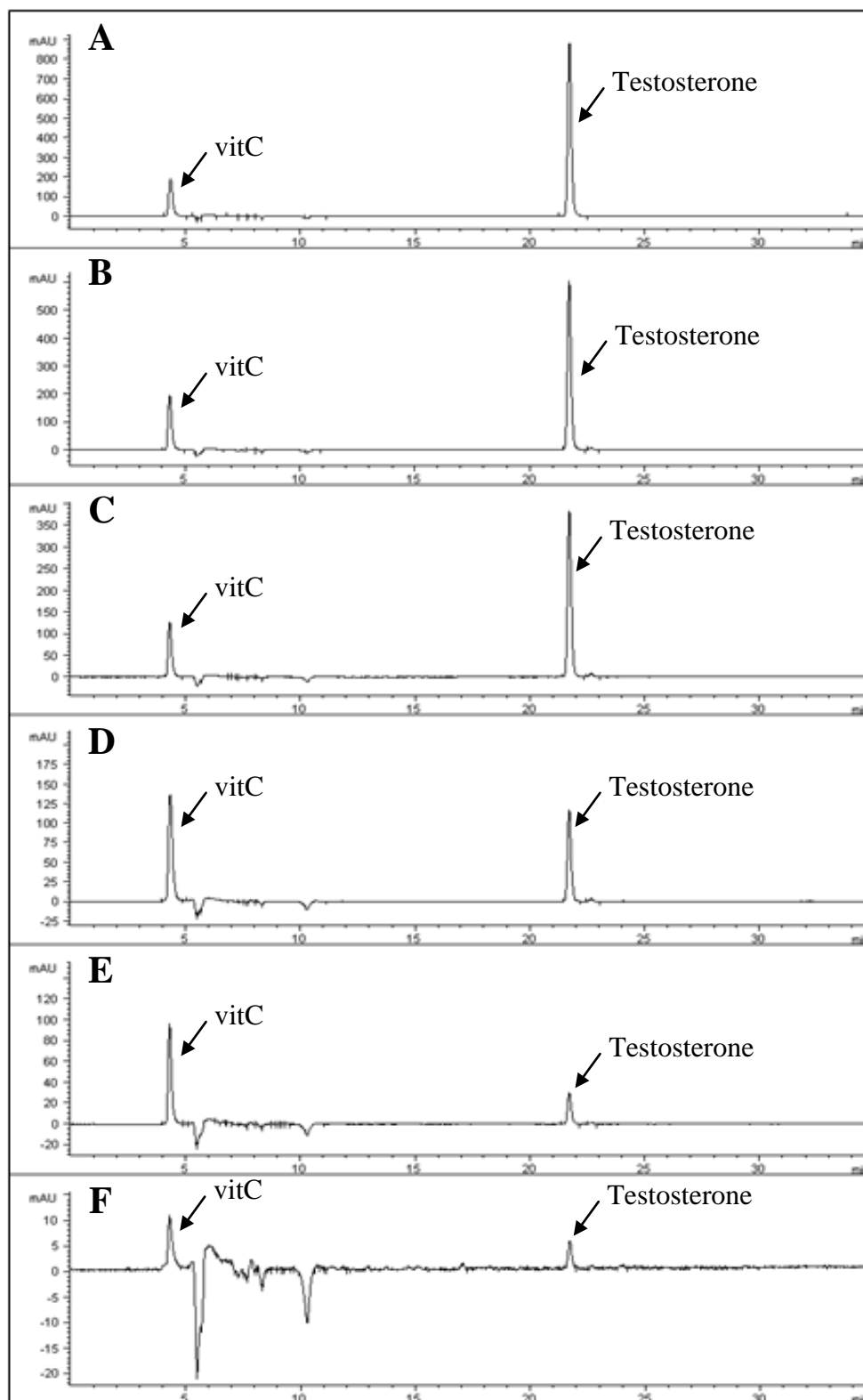## 6.4. Experiments in vitro

In the organism free radicals are permanently generated, which are crucial for various biological processes. Therefore, the attack of oxidizing ($OH^\bullet$, $O_2^{\bullet -}$, *etc.*) and reducing (e^-_{aq}, $R^\bullet$, *etc.*) free radicals on hormones and their mixtures was investigated in the frame of experiments *in vitro* with *Escherichia coli* bacteria (AB1157) in aqueous media (pH ~7.4). By application of appropriate experimental conditions (saturation of the solutions with various gases), desired kind and concentration of free radicals were obtained.

6.4.1. WsE2 and HBC

As already mentioned before, E2 was obtained as water-soluble complex with HBC, in order to be able to work in aqueous media without any additives as ethanol, which is a scavenger of a great part of the produced free radicals from water radiolysis. Thus, not only wsE2 had to be investigated, but also HBC individually, in order to enable discrimination between the effects due to HBC and E2, respectively, in the mixture of E2 and HBC (=wsE2).

6.4.1.1. Toxicity

In order to establish a suitable concentration for the following *in vitro* experiments under γ-irradiation, the effect of different concentrations of wsE2 and HBC on the bacteria was tested (114). The obtained toxicity curves of substrates HBC and wsE2, which represent the relative survival ratio of *E.coli* bacteria (N/N_0) as a function of the substrate concentration, are shown in Figure 35. HBC (curve A) exhibited toxic effects at concentrations of 3×10^{-4} mol/L and above. At lower concentrations, a proliferative effect was observed. WsE2 exhibited no toxic effect at all, but seemed to pass several maxima. In general it can be stated, that wsE2 enhanced the bacterial proliferation more at higher substrate concentration.

6.4.1.2. Survival curves under irradiation

Under appropriate experimental conditions, it is possible to produce free radicals of desired kind and concentration: In aerated solutions and in solutions saturated with N_2O, the acting primary radicals that are generated by the radiolysis of water are mainly oxidizing, whereas in solutions saturated with argon, the main part represents reducing species (cf. Table 2). Therefore, the experiments *in vitro* with *E.coli* bacteria were carried

out in aqueous media (pH ~7.4), which was saturated with air, N_2O or argon. Thus, the effect of oxidizing and reducing free radicals, respectively, on wsE2 could be investigated. The hormone transients resulting from the attack of the different types of free radicals were expected to interfere with the bacterial proliferation. Throughout all experiments a concentration of $1x10^{-4}$ mol/L wsE2 was used, which contained $3.98x10^{-4}$ mol/L HBC as a complex.

As already published (114), in Figure 36 the change of the relative survival ratio (N/N_0) of bacteria cultured in buffer (A), $3.98x10^{-4}$ mol/L HBC (B) and $1x10^{-4}$ mol/L wsE2 (C) in the presence of air is depicted as a function of absorbed radiation dose (Gy), which is proportional to the concentration of the produced free radicals. Under the applied experimental conditions, oxidizing radicals only (46% $OH^\bullet$, 54% $O_2^{\bullet-}$) operated, as $H^\bullet$ and e^-_{aq} were scavenged by O_2 and converted into peroxyl radicals (eq. 34-36).

Figure 35. Toxicity (N/N_0 ratio) of A: HBC and B: wsE2 on *Escherichia coli* bacteria (AB1157) in aqueous solution (pH ~7.4) as a function of substrate concentration (mol/L). N_0 = starting bacterial number in buffer; N = number of the bacteria incubated for 1 h in A: HBC and B: wsE2 containing media (114).
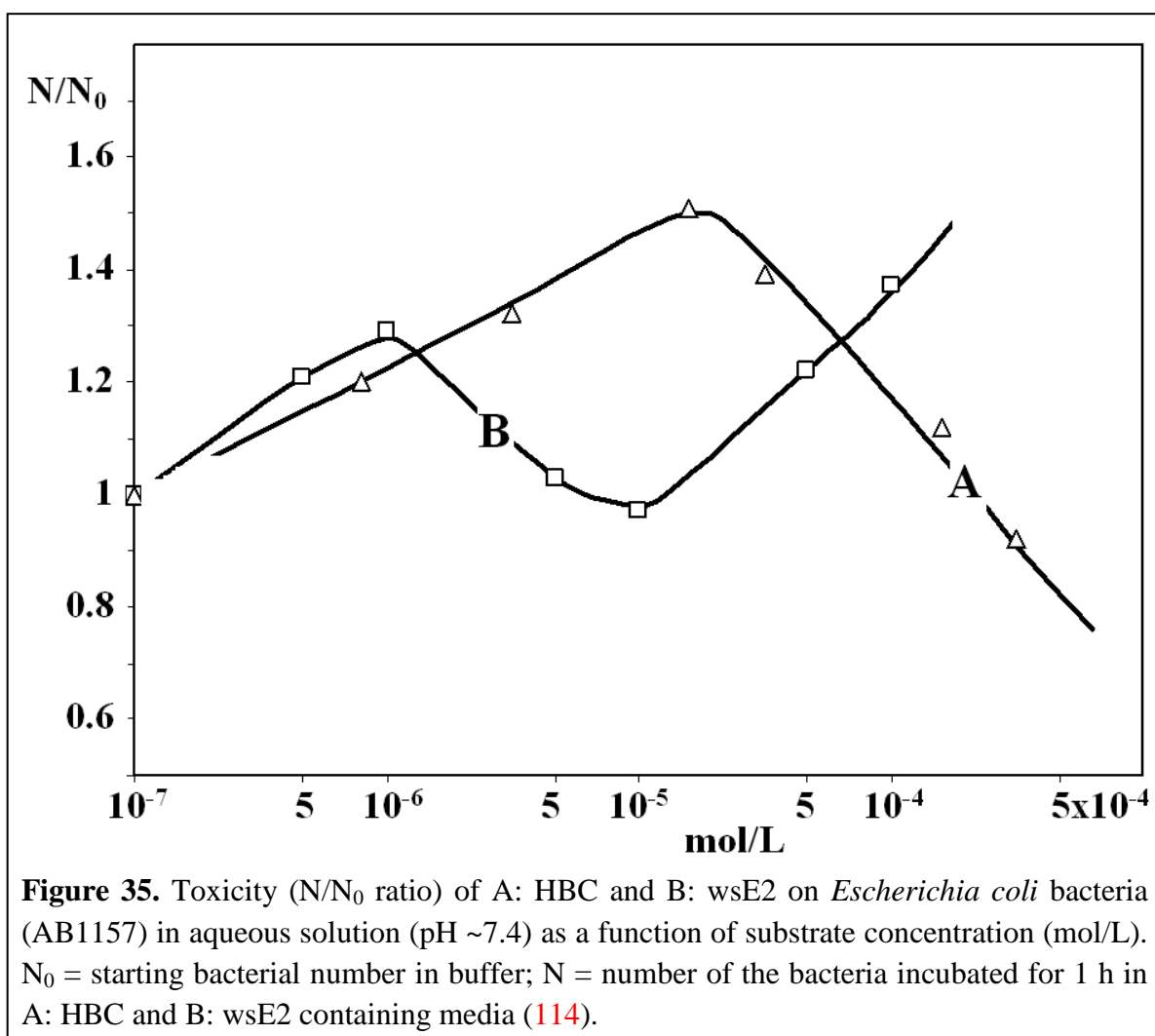
All observed ΔD_{37} values are positive (Figure 36, inset), indicating a proliferative effect of both substrates at first sight. Hereby, the transients resulting from HBC individually (curve B) exhibit an even stronger protective effect than wsE2 with a ΔD_{37} value of +110. Furthermore, a maximum is found for the N/N_0 ratio at 50 Gy, which once more points out the scavenging ability of HBC for oxidizing free radicals. The ΔD_{37} value of wsE2 is calculated to be +40 (curve C), which is much lower than the value of HBC alone. Thus, though the value is positive, it seems that E2 appears to have a cytostatic property in the present media. However, this effect is superimposed by the action of HBC.

System	ΔD_{37}
A buffer	0
B HBC	+ 110
C wsE2	+ 40

Figure 36. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous aerated media (pH ~7.4) in the presence of A: buffer; B: 3.98×10^{-4} mol/L HBC and C: 1×10^{-4} mol/L wsE2. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (114).

Figure 37. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous media saturated with N_2O (pH ~7.4) in the presence of A: buffer; B: 3.98×10^{-4} mol/L HBC and C: 1×10^{-4} mol/L wsE2. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (114).

Likewise, studies have been performed in the presence of N_2O in a broad range of radiation dose up to 600 Gy. Under these experimental conditions, all e^-_{aq} were converted into $OH^\bullet$ radicals, leading to a mainly oxidizing environment (90% $OH^\bullet$ and 10% $H^\bullet$). The obtained survival curves (N/N_0 ratio) represent mean values of several series of experiments and are presented in Figure 37 (114).

As the reaction rate constant (k) of $OH^\bullet$, the main acting species, and wsE2 is very high (k ~10^9-10^{10} L.mol^{-1}.s^{-1}), wsE2 was expected to act as a very efficient $OH^\bullet$ scavenger. This assumption was approved by the corresponding ΔD_{37} value of +250 (Figure 37, inset). From the survival curve of *E. coli* bacteria under irradiation incubated

Figure 38. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous, air-free media saturated with argon (pH ~7.4) in the presence of A: buffer; B: 3.98×10^{-4} mol/L HBC and C: 1×10^{-4} mol/L wsE2. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (114).
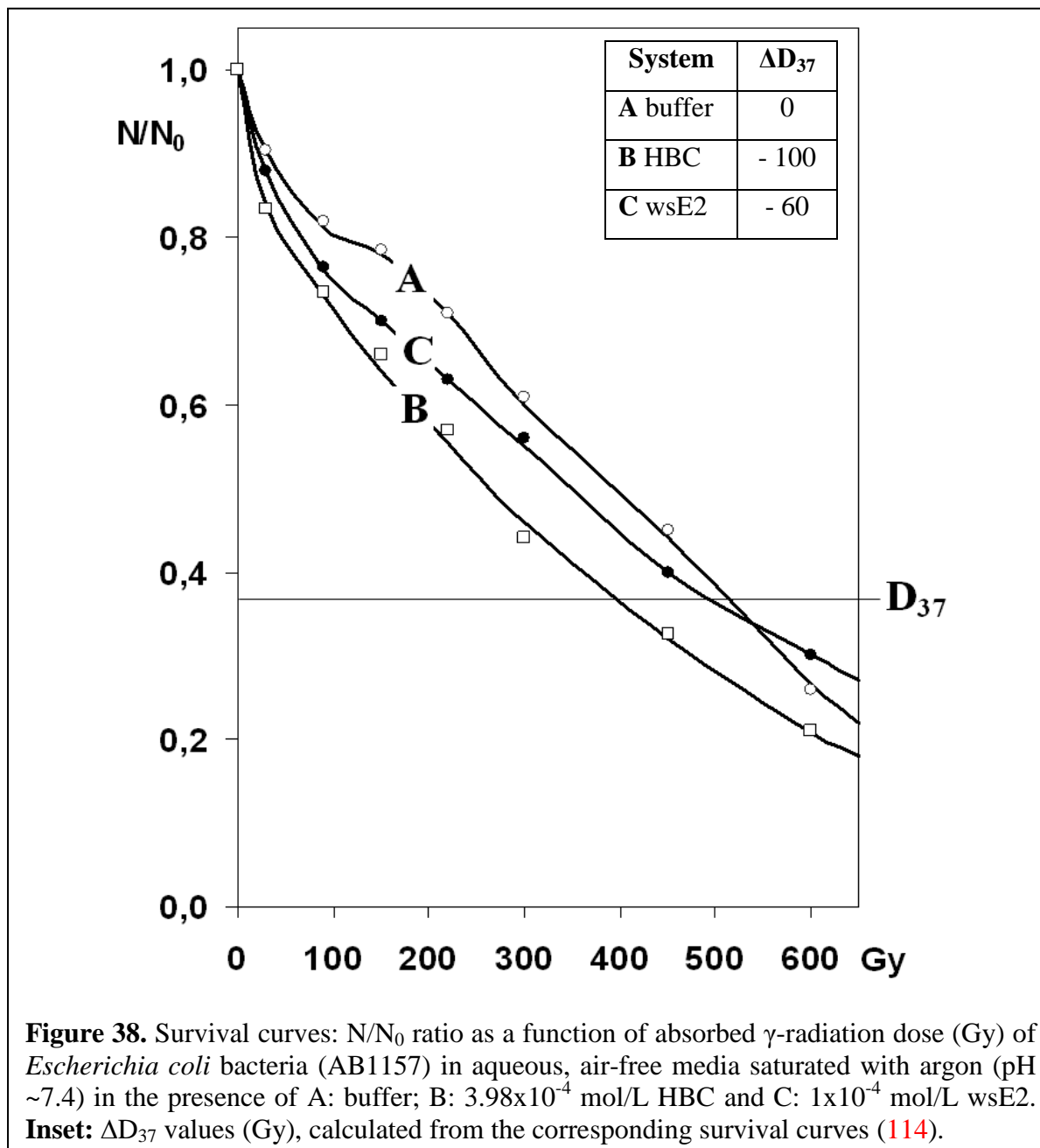
in HBC individually, a ΔD_{37} value of $+140$ was calculated. The difference in the proliferative effect of HBC alone and wsE2 can be explained by considering the reaction rate constant $k(OH^\bullet + HBC) = 7.6 \times 10^8$ L.mol^{-1}.s^{-1} (92), as $k(OH^\bullet + wsE2)$ is much higher. Comparing the ΔD_{37} values of wsE2 in aerated medium ($\Delta D_{37} = +40$) and medium saturated with N_2O ($\Delta D_{37} = +250$), it can be stated, that the protective effect of wsE2 is much higher in the latter medium, where much more $OH^\bullet$ radicals are operating.

In air-free media, *i.e.* in solutions saturated with argon, the reducing species (44% e^-_{aq}, 10% $H^\bullet$) are predominant. However, also 46% strongly oxidizing $OH^\bullet$ radicals are generated as primary products of water radiolysis, which are also involved in the

Figure 39. Toxicity (N/N_0 ratio) of wsP4 on *Escherichia coli* bacteria (AB1157) in aqueous solution (pH ~7.4) as a function of substrate concentration (mol/L). N_0 = starting bacterial number in buffer; N = number of the bacteria incubated for 1 h in wsP4 containing media (113).
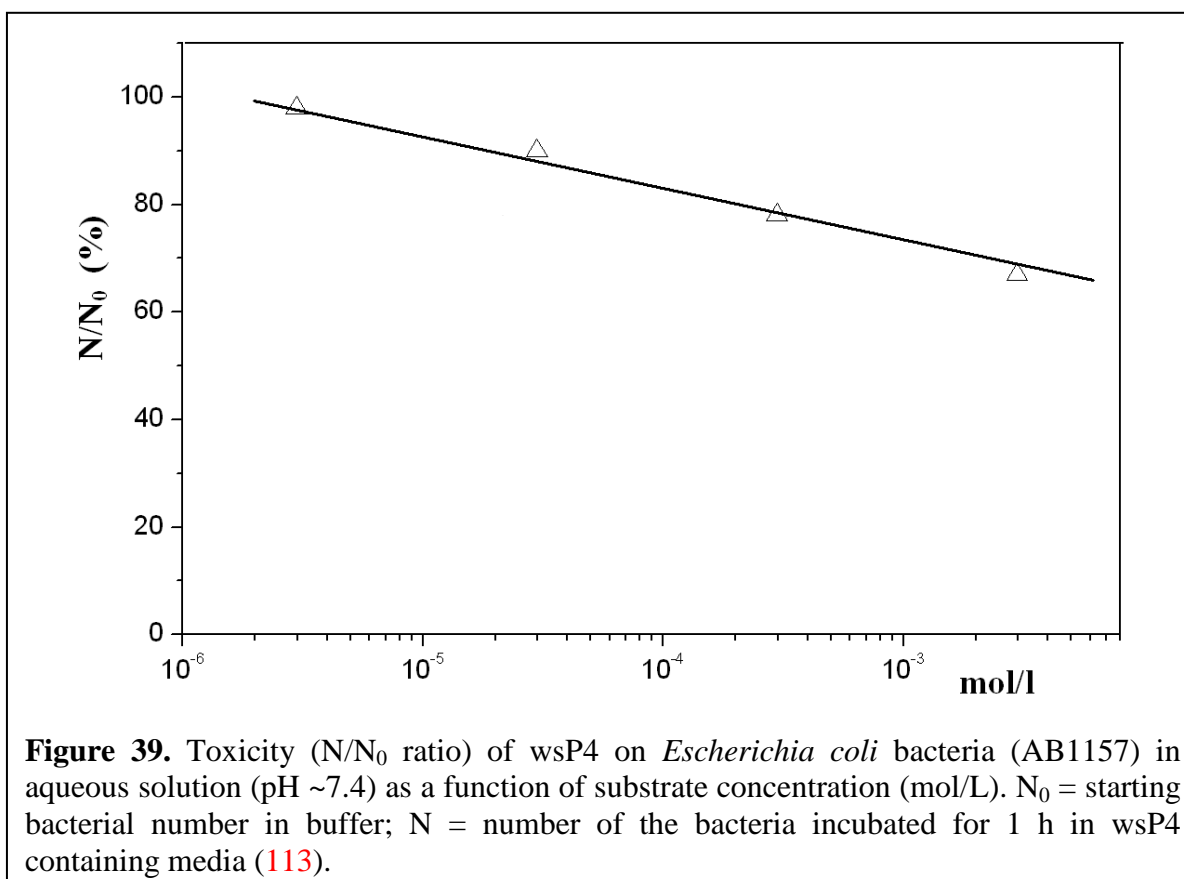
radiation-induced processes. The survival curves observed under these conditions are presented in Figure 38, showing contrary results compared with the survival curves obtained in air and N_2O. The bacteria are subject to a cytostatic effect, as both substrates HBC (curve B) and wsE2 (curve C) lead to a much faster decrease of the bacterial N/N_0 ratio under irradiation, compared to bacteria incubated solely in buffer, with a ΔD_{37} value of −100 and -60, respectively.

6.4.2. WsP4 alone and in combination with wsE2

Similar experiments *in vitro* were conducted with wsP4 alone and in mixture with wsE2, in order to examine possible mutual interactions of both hormones on the implemented bacteria *Escherichia coli* (AB1157) under γ-irradiation at pH ~7.4. WsP4 as well as wsE2 contain HBC in different ratios, in order to make them soluble in water (cf. Table 3). Therefore, HBC was investigated, as well.

6.4.2.1. Toxicity of wsP4

First of all it was of interest to determine the cytotoxicity of wsP4, which was done in a broad concentration range in aerated media (113). The mean values of several series of

experiments are presented as N/N_0 ratio in dependence of the substrate concentration (Figure 39). It can be easily seen, that the toxicity of wsP4 to *E. coli* bacteria (AB1157) increases linearly with increasing substrate concentration.
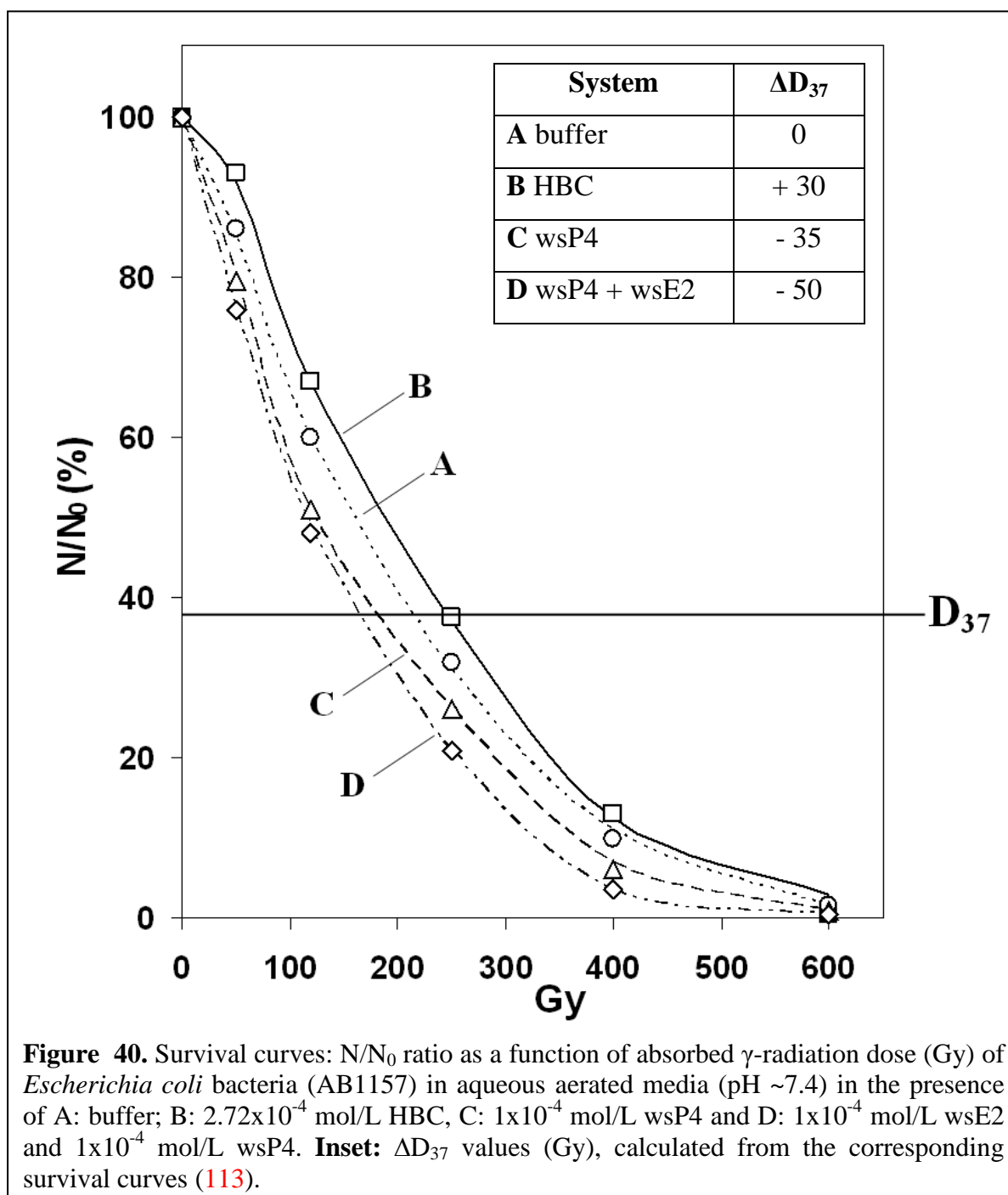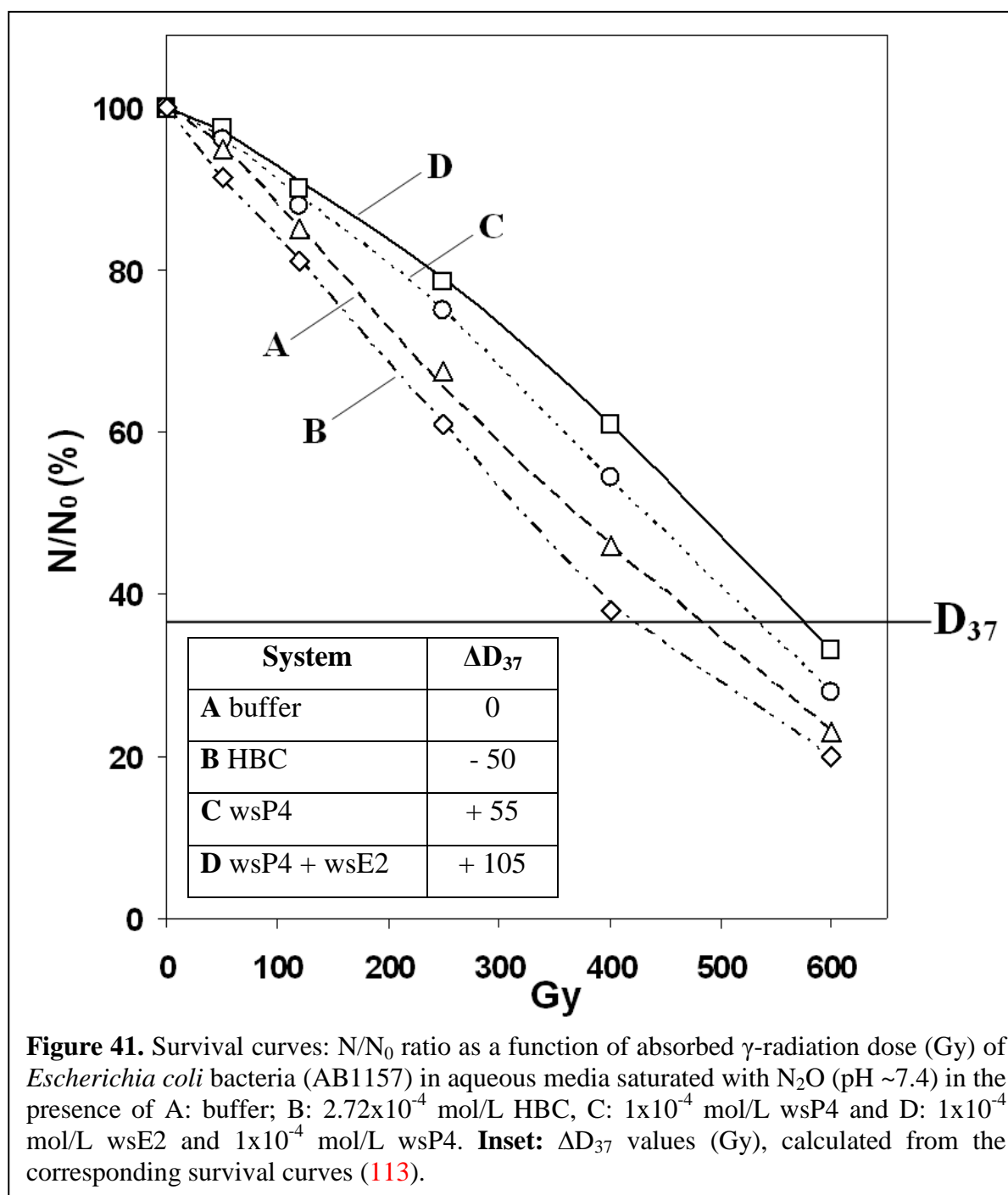
6.4.2.2.   *Survival curves under irradiation*

The concentration of $1x10^{-4}$ mol/L wsP4 (including $2.72x10^{-4}$ mol/L HBC) used for all experiments *in vitro* under irradiation was chosen according to Figure 39. Hereby, 20% of the bacteria were deactivated. In the frame of the study, survival curves with $2.72x10^{-4}$ mol/L HBC alone and a mixture of $1x10^{-4}$ mol/L wsP4 and $1x10^{-4}$ mol/L wsE2 including $6.7x10^{-4}$ mol/L HBC were also established. The obtained results in media saturated with air, N_2O and argon, respectively, are presented in Figures 40-42 as a function of the absorbed radiation dose. ΔD_{37} values, representing the radiation dose, at which N/N_0 is 0.37, were calculated and given as inset in the corresponding Figures.

Figure 40 presents the survival curves of the bacteria in aerated medium (pH ~7.4), where oxidizing radicals (46% $OH^{\bullet}$, 54% $O_2^{\bullet-}$) are produced as primary species of water radiolysis (cf. 113). $2.72x10^{-4}$ mol/L HBC (curve B) exhibits a proliferative effect on the bacteria, as it was observed before (cf. Figure 36). This fact is also illustrated by the corresponding positive ΔD_{37} value ($\Delta D_{37} = +30$), indicating once again the scavenging ability of HBC for oxidizing free radicals. In contrast, both $1x10^{-4}$ mol/L wsP4 and the mixture of $1x10^{-4}$ mol/L wsP4 and $1x10^{-4}$ mol/L wsE2 were found to exhibit a cytostatic action, whereby this effect was slightly more pronounced in the mixture of the two hormones. However, the ΔD_{37} values of -35 for wsP4 and -50 for the mixture are influenced by the protective action of HBC. Therefore, P4 and the mixture of P4 and E2 are expected to sensitize the bacteria much more towards γ-irradiation than their water-soluble counterparts. Furthermore, it is interesting to note, that though wsE2 showed a proliferative effect on *E. coli* bacteria before (cf. Figure 36), it now seems to even intensity the cytostatic property of wsP4.

The change of the bacterial survival ratio under irradiation in solutions saturated with N_2O was also studied for aqueous solutions containing the above mentioned substrates in a radiation dose range up to 600 Gy (113). Under these experimental conditions, e^-_{aq} are converted into $OH^{\bullet}$ radicals, but as the reaction rate constants of e^-_{aq} with both hormones are very high, the reaction of e^-_{aq} with hormones directly has to be considered, as well. Figure 41 presents the obtained results, which are completely different from the ones

observed in media saturated with air. Whereas 2.72×10^{-4} mol/L HBC showed a protective effect in aerated solutions, it now exhibits cytotoxic properties (Figure 41, curve B). The corresponding ΔD_{37} value is +30. The same was observed for the other two investigated substrates (1×10^{-4} mol/L wsP4 and the mixture of 1×10^{-4} mol/L wsP4 and 1×10^{-4} mol/L wsE2): Though they were found to have an anti-proliferative effect on *E. coli* bacteria in aerated medium, they now protect the cells from radiation (Figure 41, curve C and D). The mixture of wsP4 and wsE2 shows a stronger pronounced effect in this respect,

Figure 40. Survival curves: N/N$_0$ ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous aerated media (pH ~7.4) in the presence of A: buffer; B: 2.72×10^{-4} mol/L HBC, C: 1×10^{-4} mol/L wsP4 and D: 1×10^{-4} mol/L wsE2 and 1×10^{-4} mol/L wsP4. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (113).

Figure 41. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous media saturated with N_2O (pH ~7.4) in the presence of A: buffer; B: 2.72×10^{-4} mol/L HBC, C: 1×10^{-4} mol/L wsP4 and D: 1×10^{-4} mol/L wsE2 and 1×10^{-4} mol/L wsP4. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (113).

which is illustrated by $\Delta D_{37} = +55$ for wsP4 and $\Delta D_{37} = +105$ for the mixture. Again it has to be stated, that these results are superimposed by the action HBC. Therefore, P4 and E2 are expected to exhibit much stronger pronounced protective effects under these experimental conditions.

In order to examine the effect of reducing free radicals on wsP4 and its mixture with wsE2, experiments *in vitro* in air-free solutions were conducted (113), where reducing (44% e^-_{aq}, 10% $H^\bullet$) and oxidizing free radicals (46% $OH^\bullet$) are generated as primary

products of water radiolysis. The obtained survival curves (N/N_0 ratio) are depicted in Figure 42 as a function of the absorbed radiation dose (Gy). As observed before (Figure 38, curve B), the transients of HBC resulting from the attack of the acting free radicals under the given experimental conditions lead to a cytotoxic effect on the implemented bacterial strain ($\Delta D_{37} = -30$). On the other hand, wsP4 exhibits a protective effect ($\Delta D_{37} = +50$), which is even enforced, when the bacteria are incubated in mixture with wsE2 ($\Delta D_{37} = +110$). It is interesting to note, that wsE2 alone showed an anti-proliferative effect under the same conditions (Figure 38, curve C).

System	ΔD_{37}
A buffer	0
B HBC	- 30
C wsP4	+ 50
D wsP4 + wsE2	+ 110

Figure 42. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in air-free, aqueous media saturated with argon (pH ~7.4) in the presence of A: buffer; B: 2.72×10^{-4} mol/L HBC, C: 1×10^{-4} mol/L wsP4 and D: 1×10^{-4} mol/L wsE2 and 1×10^{-4} mol/L wsP4. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves (113).
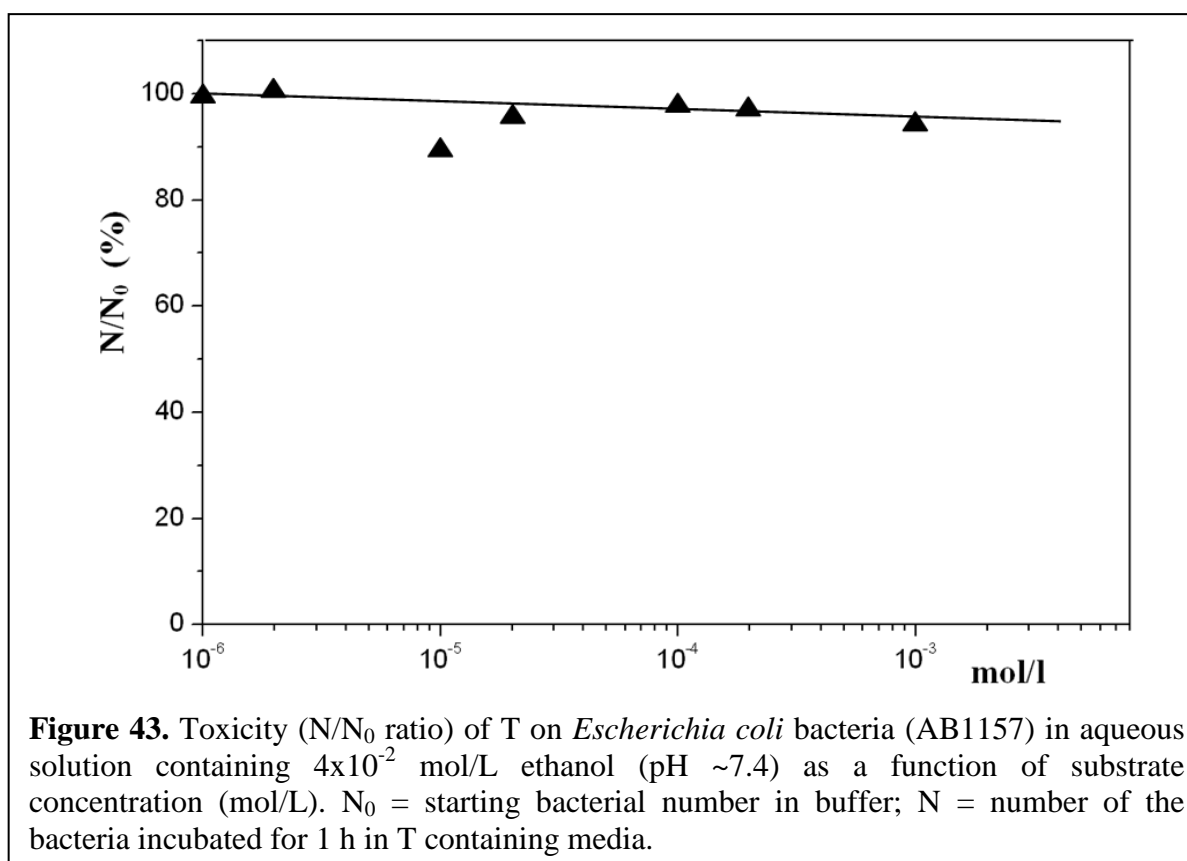
6.4.3. T and VitC

Due to the already mentioned postulation, that a potent electron donor like vitC could contribute to a possible regeneration of hormones, such as T, the effect of oxidizing and reducing free radicals on T, vitC and their mixture was investigated by experiments *in vitro* with *Escherichia coli* bacteria (AB1157) in a medium containing 4×10^{-2} mol/L ethanol with a pH of ~7.4. Since ethanol has a strong scavenging ability for all primary free radicals except e^-_{aq}, other kinds and concentrations of free radicals than the ones mentioned for the experiments with water-soluble hormones were produced in solutions saturated with air, N_2O or argon by water radiolysis (eqs. 38,39).
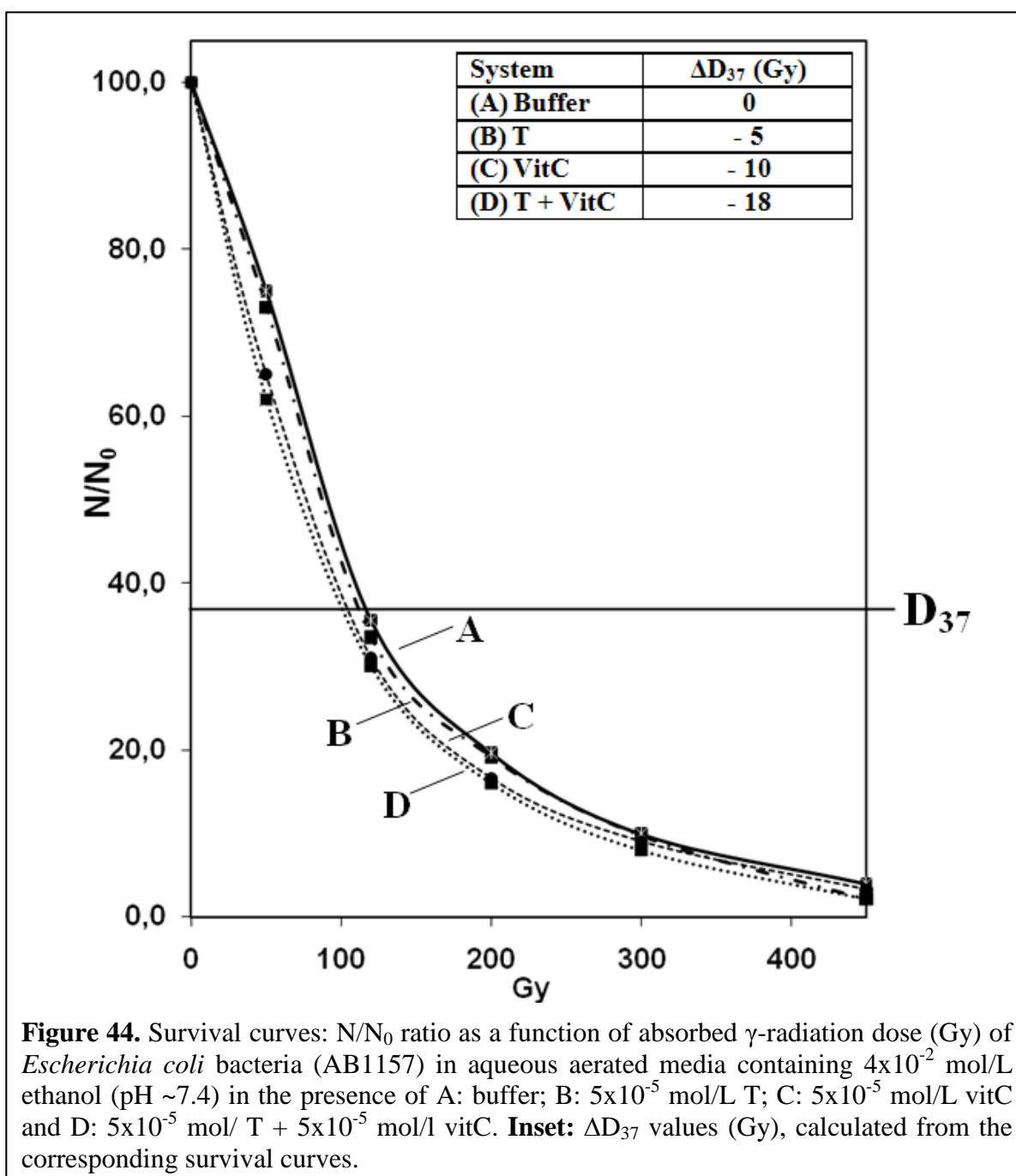
6.4.3.1. Toxicity of T

Firstly, it was necessary to investigate the cytotoxicity of T in a broad range of concentration from 1×10^{-6} mol/L to 1×10^{-3} mol/L in aqueous, aerated solution containing 4×10^{-2} mol/L ethanol, in order to establish a suitable concentration for the subsequent experiments under irradiation. The obtained change of the N/N_0 ratio is presented in Figure 43 as a function of the substrate concentration. Hereby, with increasing substrate concentration, a slight decline of the number of viable cells is observed.

Figure 43. Toxicity (N/N_0 ratio) of T on *Escherichia coli* bacteria (AB1157) in aqueous solution containing 4×10^{-2} mol/L ethanol (pH ~7.4) as a function of substrate concentration (mol/L). N_0 = starting bacterial number in buffer; N = number of the bacteria incubated for 1 h in T containing media.
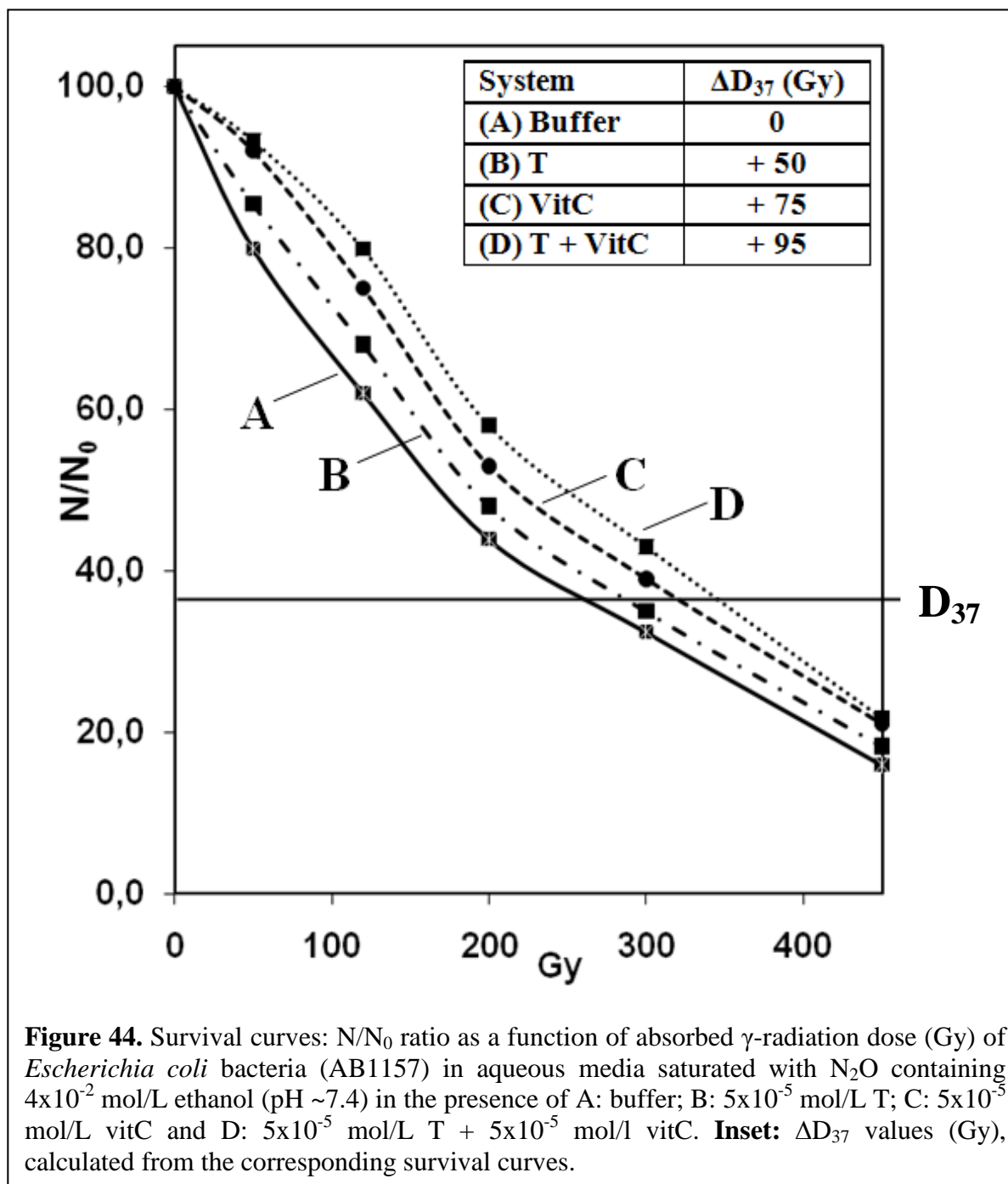
6.4.3.2.   *Survival curves under irradiation*

The change of the bacterial survival ratio in solutions saturated with air, N_2O or argon, under γ-irradiation was studied with 5×10^{-5} mol/L T, 5×10^{-5} mol/L vitC and their mixture (5×10^{-5} mol/L T and 5×10^{-5} mol/L vitC) in a dose range up to 450 Gy. All solutions also contained 4×10^{-2} mol/L ethanol, as the hormones are not soluble in pure water.

Figure 44 presents the survival curves of the bacteria in aerated medium (pH ~7.4), where oxidizing radicals are operating. The curves are all very close to each other. However, T intermediates resulting from attack of oxidizing free radicals show weak

Figure 44. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous aerated media containing 4×10^{-2} mol/L ethanol (pH ~7.4) in the presence of A: buffer; B: 5×10^{-5} mol/L T; C: 5×10^{-5} mol/L vitC and D: 5×10^{-5} mol/ T + 5×10^{-5} mol/l vitC. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves.

Figure 44. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in aqueous media saturated with N_2O containing 4×10^{-2} mol/L ethanol (pH ~7.4) in the presence of A: buffer; B: 5×10^{-5} mol/L T; C: 5×10^{-5} mol/L vitC and D: 5×10^{-5} mol/L T + 5×10^{-5} mol/l vitC. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves.

cytostatic properties. Bacteria incubated with vitC and the mixture of T and vitC are also subject to a cytostatic effect, whereby the mixture shows a more pronounced impact. Of course, the ΔD_{37} values, which were calculated from the corresponding curves, are also very close with a value of -5 for T, -10 for vitC and -18 for their mixture.

Likewise, experiments in solutions saturated with N_2O were conducted (Figure 44), where mainly reducing species are produced, as $OH^{\bullet}$ and $H^{\bullet}$ are scavenged by ethanol and converted into ethanol radicals, which act as reducing species. Under these experimental
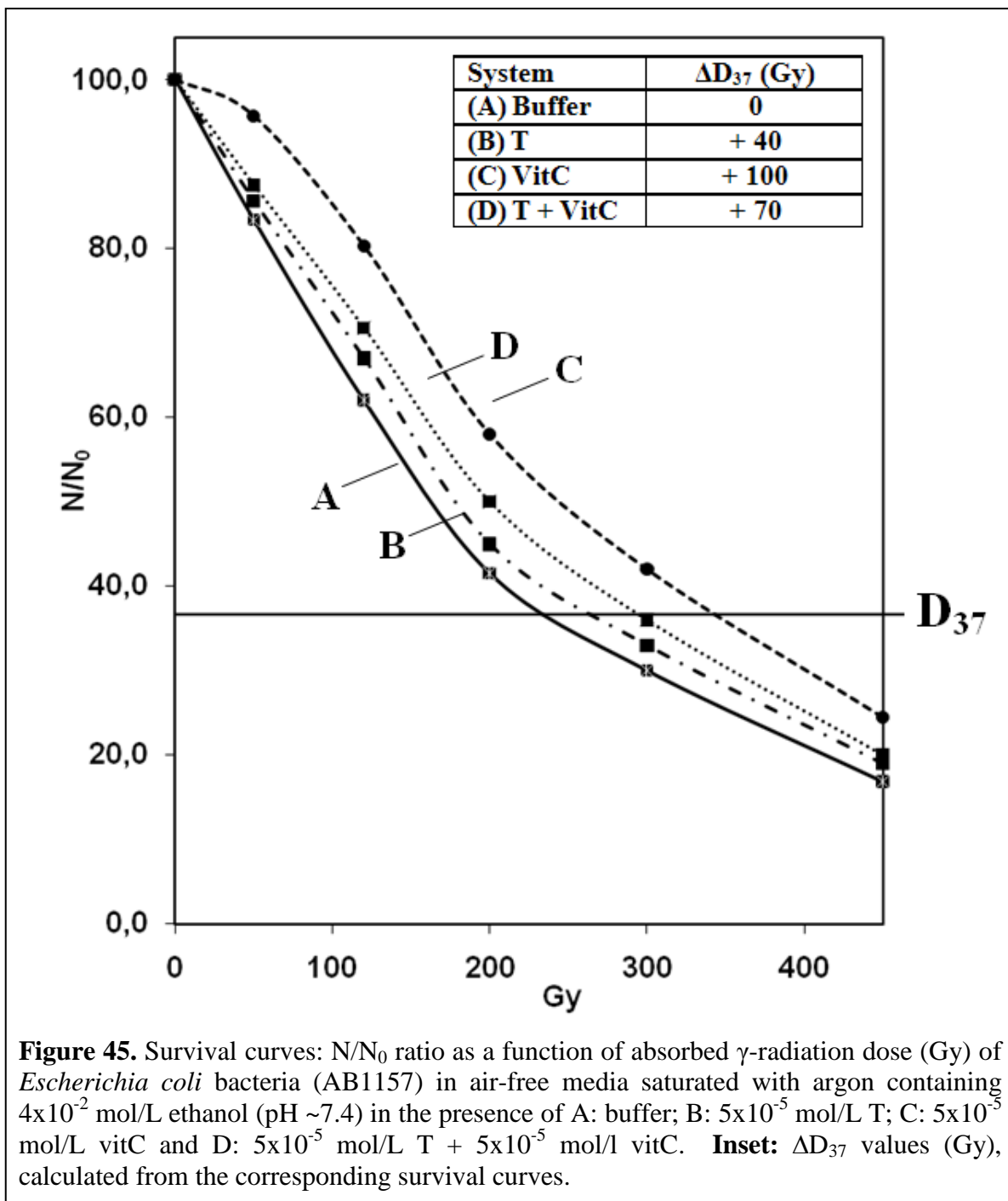
Figure 45. Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of *Escherichia coli* bacteria (AB1157) in air-free media saturated with argon containing 4×10^{-2} mol/L ethanol (pH ~7.4) in the presence of A: buffer; B: 5×10^{-5} mol/L T; C: 5×10^{-5} mol/L vitC and D: 5×10^{-5} mol/L T + 5×10^{-5} mol/l vitC. **Inset:** ΔD_{37} values (Gy), calculated from the corresponding survival curves.

conditions, T (ΔD_{37} = +50) as well as vitC (ΔD_{37} = +75) exhibit a protective effect under γ-irradiation (Figure 44, curve B and D). However, when T is combined with vitC, the protective action of T is even intensified (curve D). This is also illustrated by the rather high corresponding ΔD_{37} value of +95.

Figure 45 presents the survival curves of the bacteria in medium saturated with argon (pH ~7.4), where only reducing species are acting (e^-_{aq}, ethanol radicals), as a function of the absorbed radiation dose. Again, all substrates show positive ΔD_{37} values, indicating a

proliferative action of the intermediates resulting from the attack of the free radicals on the bacteria. The corresponding ΔD_{37} values are +40 for T, +100 for vitC and +70 for their mixture. It is interesting to note, that the mixture doesn't exhibit the most pronounced effect under these experimental conditions, as it was before in the presence of air (Figure 43) and N_2O (Figure 44).

Comparing the data obtained in various media (Figures 43-45), it can be concluded, that all three substrates exhibit anti-proliferative effects on *E. coli* bacteria under the attack of oxidizing radicals, but proliferative effect when they are subject to reducing species.

7. DISCUSSION

7.1. Formation of hormone associates

For all investigated hormones (T, P4, wsE2, wsP4, GEN) it was observed that the extinction coefficient values at λ=254 nm (ε_{254} values) were decreasing with increasing substrate concentration (98,99,112,113), though the ε value of a compound is usually constant, regardless of the concentration, as already mentioned (Lambert-Beer's Law). The obtained values for 1×10^{-5} mol/L and 5×10^{-5} mol/L T and 1×10^{-5} mol/L and 5×10^{-5} mol/L P4 are presented in inset of Figure 23. This observation indicates the formation of hormone associates (hormone clusters), which are preferentially built with higher substrate concentrations. In the meantime, the same effects were found for other hormones, such as 4-OHE1 (48), 17OHP (49) and E1 (50).

7.2. $Q(e^-_{aq})$ values decrease with increasing hormone concentration

In the experiments studying the emission of electrons from various hormones as a result of excitation to their singlet states by UV-irradiation, it was found that with increasing substrate concentration (mol/L) the $Q(e^-_{aq})$ values are decreasing (98,99,112,113). Therefore, the obtained $Q(e^-_{aq})$ values, taking GEN as representative example for hormones, were plotted as a function of the substrate concentration, whereby a strict linear dependence was found (Figure 46). Furthermore, the number of electron emitting GEN molecules (in %) was calculated and is given as inset in Figure 46. It decreases from 36% for 2.5×10^{-5} mol/L GEN to 4% for 1×10^{-4} mol/L GEN.

There are several explanations for the observed effect of decreasing $Q(e^-_{aq})$ values with increasing substrate concentration (98,99,112,113). First, the above mentioned hormone associates have to be considered, as the hormone molecules in the ground state, which are within a hormone cluster, are able to consume electrons emitted from other hormone molecules. As a consequence, it is postulated, that with increasing hormone concentration, a higher number of hormone associates is formed, resulting in an increased "self-consumption" of e^-_{aq}. Thus, $Q(e^-_{aq})$ values diminish. It should be also noted, that the dissimination of the absorbed UV quanta is probably not homogenously distributed within the hormone associates, causing energy quenching. Further, competitive reaction mechanisms of chloroethanol and the hormones for e^-_{aq} ($k(e^-_{aq}+ClC_2H_4OH) = 4 \times 10^8$ L.mol^{-1}.s^{-1} (92), $k(e^-_{aq}+$hormone) $\sim 4 \times 10^9$ up to 2×10^{10} L.mol^{-1}.s^{-1}), contribute to the observed effect.
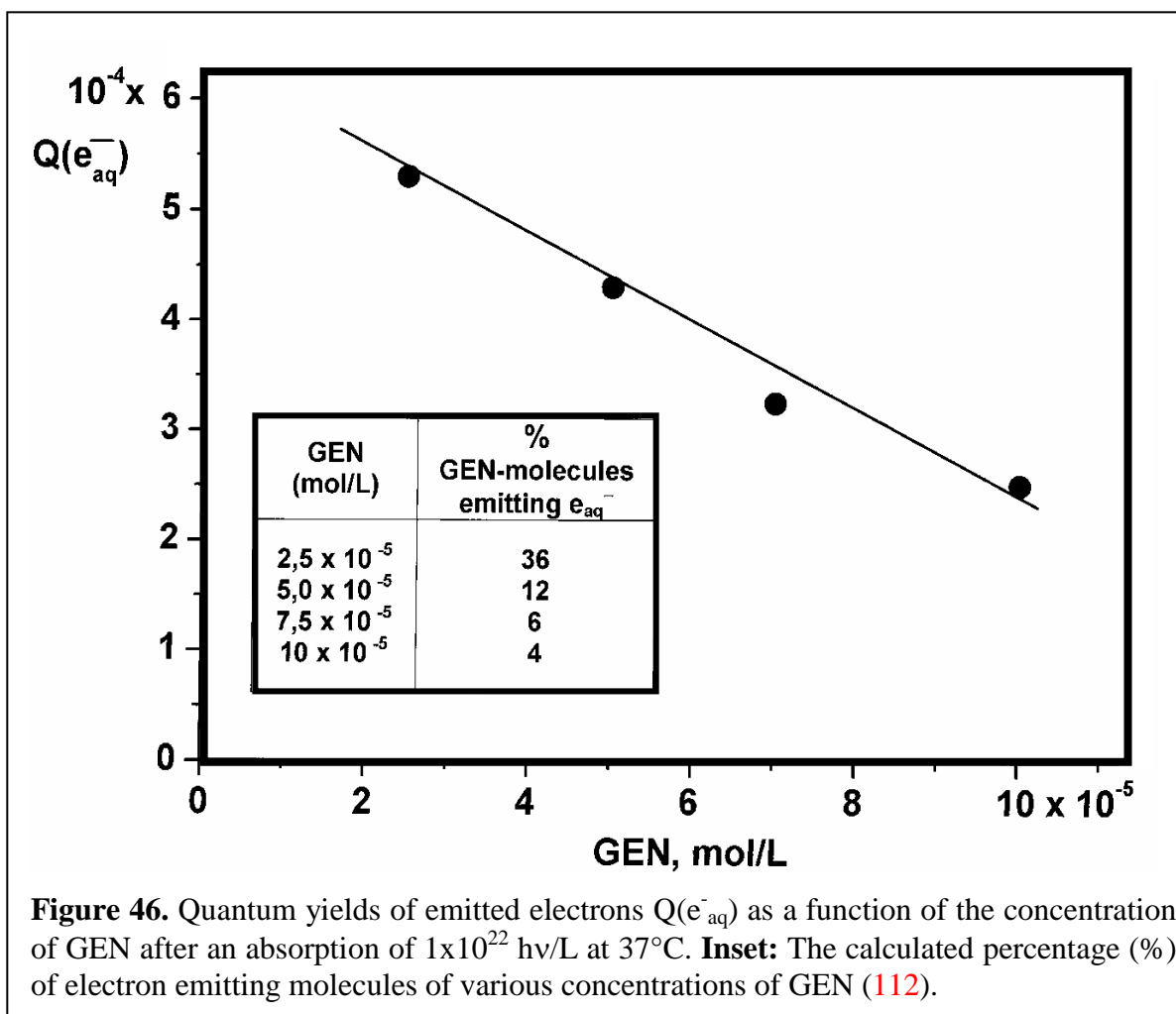
Figure 46. Quantum yields of emitted electrons $Q(e^-_{aq})$ as a function of the concentration of GEN after an absorption of 1×10^{22} hv/L at 37°C. **Inset:** The calculated percentage (%) of electron emitting molecules of various concentrations of GEN (112).

Alternatively, the diminution of $Q(e^-_{aq})$ values with increasing substrate concentration may also be due to photolytic products, which are formed as a result of electron emission from hormones. They are also very likely able to consume e^-_{aq} emitted from hormones.

7.3. UV-excitation of hormones is followed by multi-stage processes
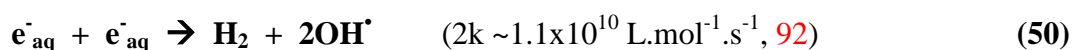
The curves of electron emission from various hormones and HBC show a wave-like course, exhibiting several maxima. This was observed for all UV-excited substrates: 5×10^{-5} mol/L T, 5×10^{-5} mol/L P4 and the mixture of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4 (Figure 24), 1×10^{-4} mol/L HBC (Figure 26), 1×10^{-4} mol/L wsE2 and 1×10^{-4} mol/L wsP4 (Figure 27) and their mixture (Figure 28). The observed second, third, *etc.* maxima indicate that hormone radicals are formed as a result of the ejection of electrons, which give rise to various products, which may be able to emit or to consume electrons, as well. Depending on the availability of certain reaction partners present in the medium, the formation of more or less carcinogenic products is expected.

7.4. Temperature influences the electron emission ability of hormones

The emission of electrons from 1×10^{-5} mol/L T and 5×10^{-5} mol/L GEN in polar media was tested for its dependence of the temperature (99,112), as an impact in this respect has been previously found for E2 (47) and other organic compounds (52). The obtained results are presented in Figures 22 and 25. It can be easily seen, that the established electron yields increase with rising temperature in both cases. The calculated initial $Q(e^-_{aq})$ values of GEN at different temperatures are given as inset in Figure 22, and point out the impact of the temperature on the ability of this substrate to emit electrons, once more. A possible explanation for this effect might be the fact that with rising temperature the emission of electrons results from a higher vibrational level of the singlet state (cf. Figure 7).

7.5. Electron emission of GEN compared with E2

GEN, a phytohormone with estrogenic properties, and E2 are very similar in their molecular structures (Figure 5). For 1×10^{-4} mol/L GEN in aqueous medium at 37°C, a $Q(e^-_{aq})$ value of 2.5×10^{-4} was observed (Figure 21) (112), which is much lower compared to that of E2, which is 0.91×10^{-2} (47) under the same experimental conditions, but in a solvent medium containing 40% water and 60% ethanol. As the polarity of water is much stronger than that of ethanol, the composition of the solvent medium is an important factor for the stability of the solvated electrons. In aqueous solutions, e^-_{aq} has a lifetime of ~250 μs, whilst in alcohol the life span is essentially shorter. Thus, in the latter case, the electrons are scavenged by chloroethanol to a lesser extent, which was used as scavenger throughout all experiments concerning the emission of electrons. Electrons are also able to react with each other (eq. 50).

$$\mathbf{e^-_{aq} \; + \; e^-_{aq} \; \rightarrow \; H_2 \; + \; 2OH^\bullet} \qquad (2k \sim 1.1 \times 10^{10} \text{ L.mol}^{-1}.\text{s}^{-1}, 92) \qquad \mathbf{(50)}$$

However, the observed effect can be explained by the competitive reactions of the GEN molecule. In contrast to E2, which mainly emits electrons, predominantly from the OH-group of the aromatic ring A, GEN possesses electron emitting as well as consuming functional groups. Similar to E2, GEN has OH-groups on aromatic rings, which are emitting electrons, when excited in their singlet states with UV-irradiation of a wavelength of 254 nm. Taking phenol as an example for aromatic rings carrying an OH-

group, the corresponding $Q(e^-_{aq})$ value is ~3×10^{-2} (92). At the same time, the carbonyl group (C=O) of GEN as well as the oxygen in the middle ring consume electrons with reaction rate constants of $k(CO + e^-_{aq}) = 1.7 \times 10^9$ L.mol^{-1}.s^{-1} and $k(O_2 + e^-_{aq}) = 1.9 \times 10^{10}$ L.mol^{-1}.s^{-1}, respectively (92,100). As these reaction rate constants are rather high, the emitted electrons are consumed by GEN itself to a large extent. For comparison, the reaction rate constant of the electron scavenger chloroethanol is smaller by one to two orders of magnitude (k ~4×10^8 L.mol^{-1}.s^{-1}, 92).
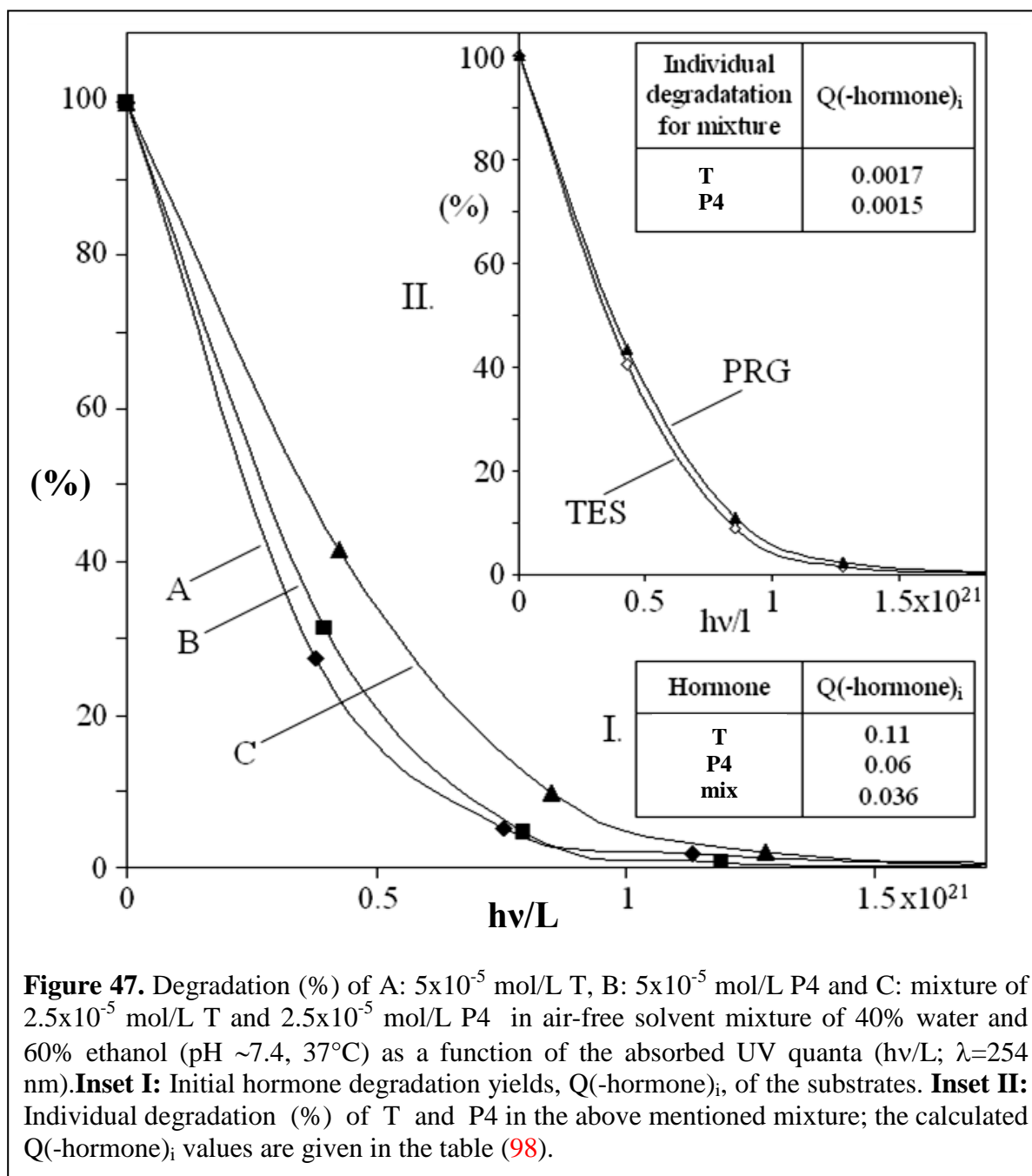
7.6. Testosterone

7.6.1. Effect of P4 on the electron emission and degradation of T

In studies concerning the electron emission from UV-excited 5×10^{-5} mol/L T, 5×10^{-5} mol/L P4 and the mixture of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4 in a solvent consisting of 40% water and 60% ethanol, the involvement of multi-stage processes can be easily seen, as all obtained curves exhibit several maxima (Figure 24). Simultaneously, HPLC-studies were conducted, the results of which are presented in Figures 29-31. Hereby, highest product yields and most degradation products were found in the UV-irradiated mixture of T and P4 (Figure 31), indicating that the two hormones possibly form unstable complexes.

Out of the data obtained by HPLC-analyses, the degradation (%) of the hormones and their mixture was calculated (98). The results are depicted in Figure 47 as a function of the absorbed UV dose (hν/L). The initial degradation yields, $Q(\text{-hormone})_i$, were calculated from the tangent-line of the corresponding curve (insets, Figure 41). At an absorbed UV dose of 1×10^{21} hν/L, the hormones are already decomposed in large part. Considering that the first peak of electron emission from the hormones and their mixture (Figure 24) occurs at the same absorbed UV dose, it can be stated that only the first peak belongs exclusively to the electron emission from the hormones themselves. Therefore, observed second, third *etc.* peaks are only partly a result of the electron ejection of the individual hormones, as electrons emitted by metabolites play a decisive role in this respect.

Comparing the electron emission of T and P4 (Figure 24, inset), $Q(e^-_{aq})$ from T turns out to be 3.6 times higher ($Q(e^-_{aq}) = 2.5 \times 10^{-2}$ for peak 1 of T and $Q(e^-_{aq}) = 0.69 \times 10^{-2}$ for peak 1 of P4). For the hormone mixture (curve C), the value of peak (1) is even ten times lower. This fact can be explained by the different molecular structures of the hormones

Figure 47. Degradation (%) of A: 5×10^{-5} mol/L T, B: 5×10^{-5} mol/L P4 and C: mixture of 2.5×10^{-5} mol/L T and 2.5×10^{-5} mol/L P4 in air-free solvent mixture of 40% water and 60% ethanol (pH ~7.4, 37°C) as a function of the absorbed UV quanta (hν/L; λ=254 nm).**Inset I:** Initial hormone degradation yields, Q(-hormone)$_i$, of the substrates. **Inset II:** Individual degradation (%) of T and P4 in the above mentioned mixture; the calculated Q(-hormone)$_i$ values are given in the table (98).

(98). T carries a hydroxyl group favouring the electron emission at position 17 of ring D, whilst P4 has a carbonyl group at the same position, which consumes a part of the emitted e^-_{aq}. Additionally, both hormones have a double bond at positions 4–5 of ring A, which represents another source for electron emission.

Eq. 51a illustrates the ejection of electrons from position 17 of ring D of T, resulting in the formation of a T radical (T$^\bullet$). As by-product, H$^+$ is formed, which is responsible for the observed decrease of the pH with increasing radiation dose. Furthermore, it is shown, that the resulting hormone radicals can react with various species (R$^\bullet_x$) present in the

media, leading to the formation of metabolites (98). In contrast, eq. 51b illustrates the ejection of electrons from the π-electron structure of the double bond of ring A of T, leading to the formation of a radical cation (T$^{\bullet+}$).
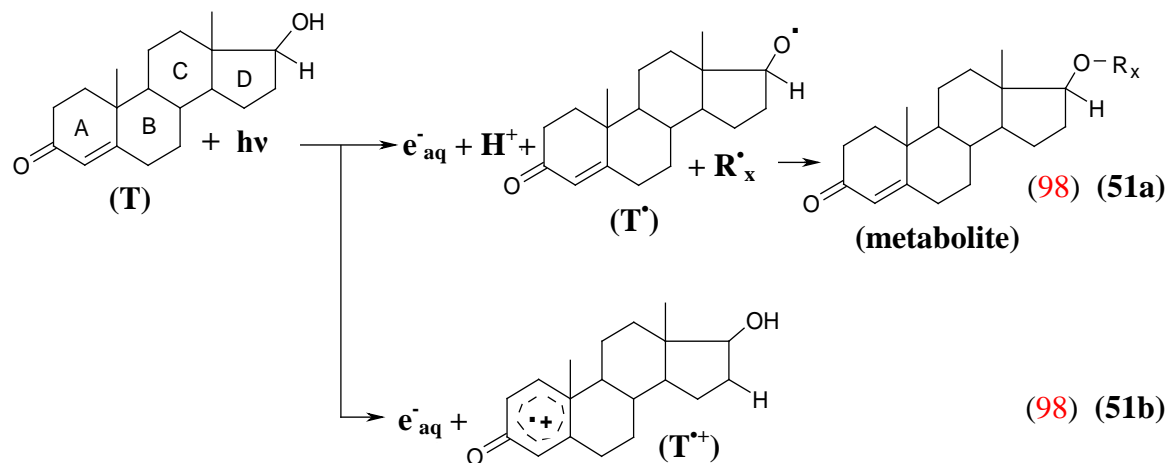
Due to the fact, that T is dissolved in a mixture containing ethanol, possible metabolite formation can occur with the involvement of ethanol (eq. 52).

$$T^{\bullet} + {}^{\bullet}C_2H_4OH \rightarrow T\text{-}C_2H_4OH \text{ (adduct)} \qquad (99) \ (52)$$

As P4 also possesses a double bond on its ring A, a similar reaction to eq. 51b occurs, which is shown by eq. 53.
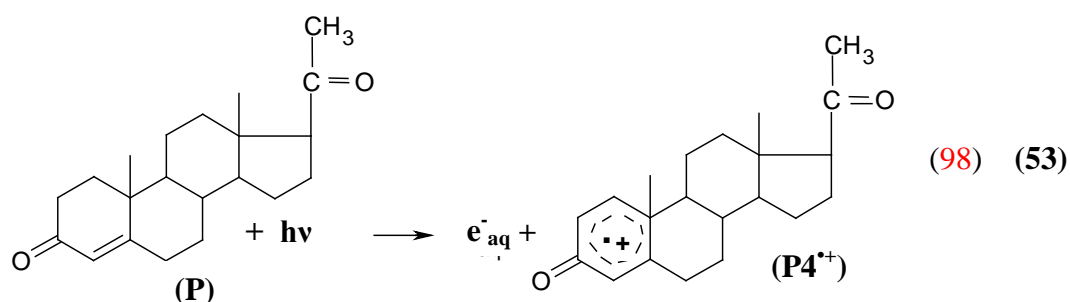
The produced radical cations of T and P4 (T$^{\bullet+}$ and P4$^{\bullet+}$) can be regenerated by reaction with the emitted electrons and, therefore, consume a part of e$^-_{aq}$. In addition, they are able to react with water, leading to the production of OH$^{\bullet}$ radicals, which may add to the hormone degradation to a very small extent, because the OH$^{\bullet}$ radicals are scavenged by ethanol (eq. 38).

$$P4^{\bullet+} \; + \; H_2O \; \rightarrow \; P4 \; + \; H^+ \; + \; OH^{\bullet} \tag{54}$$

As both hormones possess functional groups, which are consuming electrons, a part of the emitted electrons is scavenged by the hormones in ground state. The possible reaction mechanisms in this respect for the carbonyl groups of ring A and on position 17 of ring D of P4 are presented by eqs. 55a and 55b, respectively. Upon reaction of the resulting radical anions ($P4^{\bullet-}_1$ and $P4^{\bullet-}_2$) with various species ($R^{\bullet}_x$) present in the medium corresponding metabolites are formed.
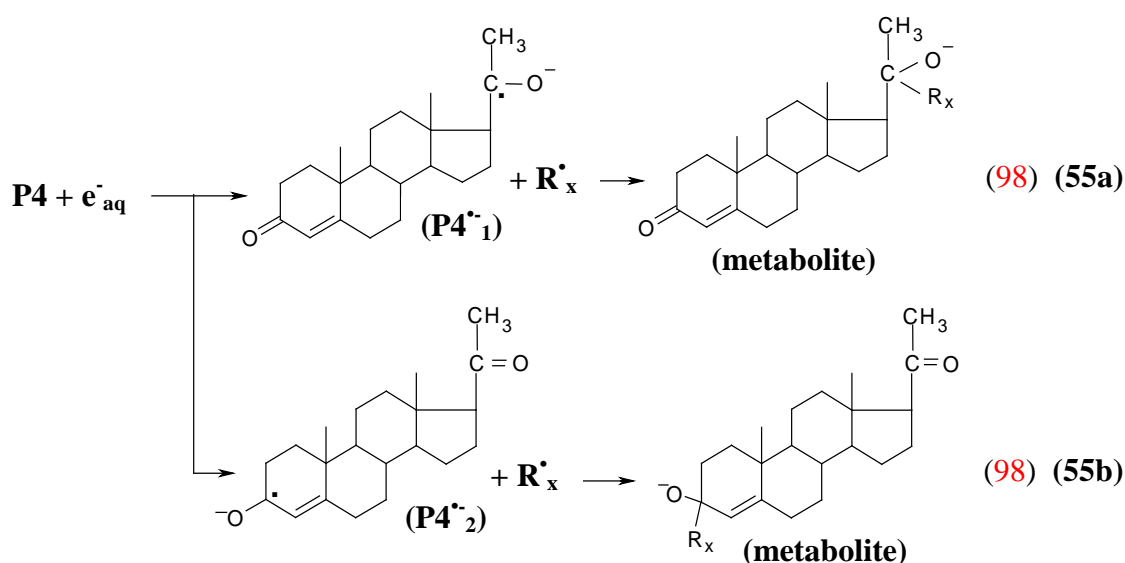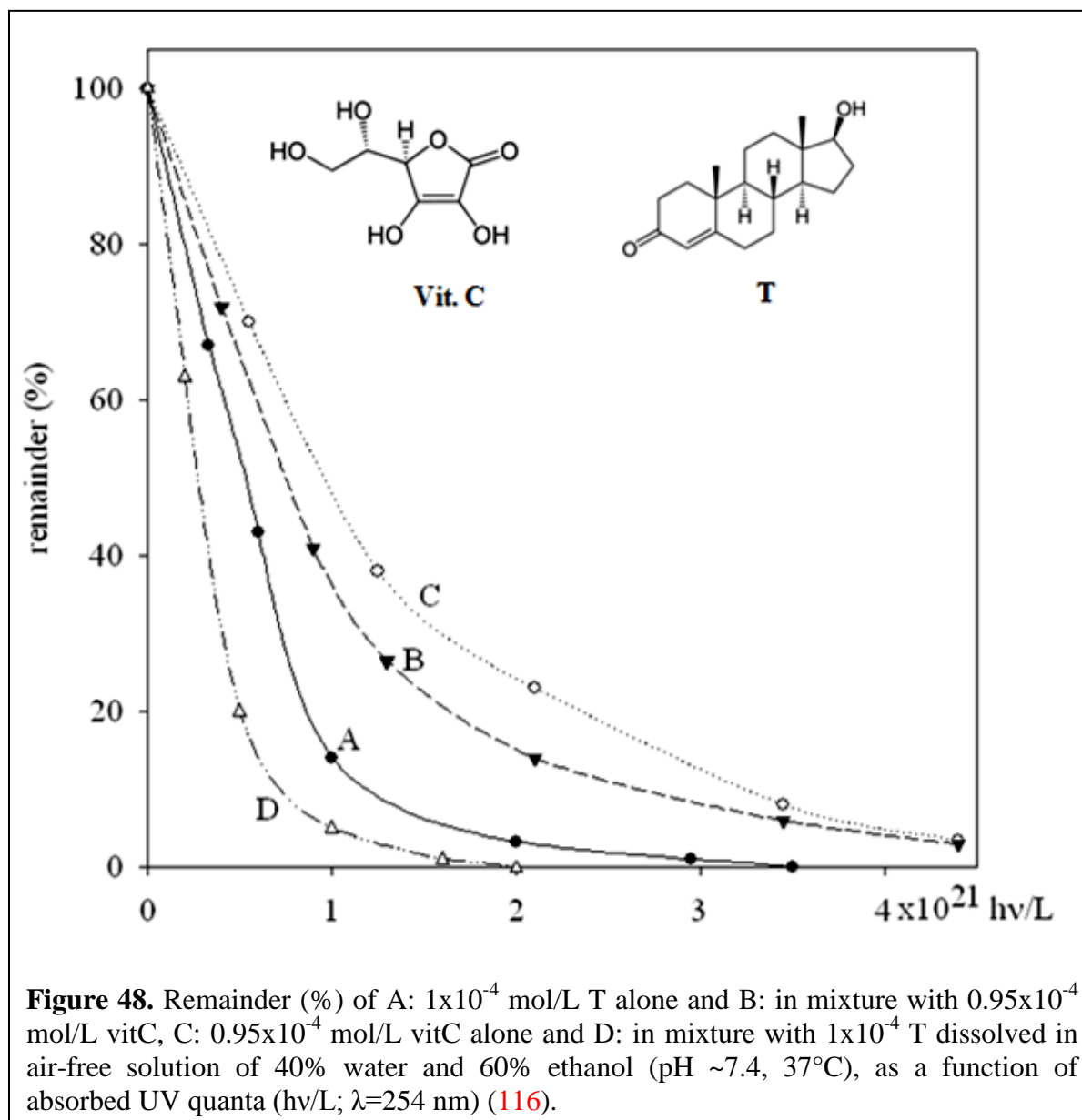

A similar reaction to eq. 55b occurs also with T as a consequence of the identical structure of ring A, resulting in a radical anion of T.
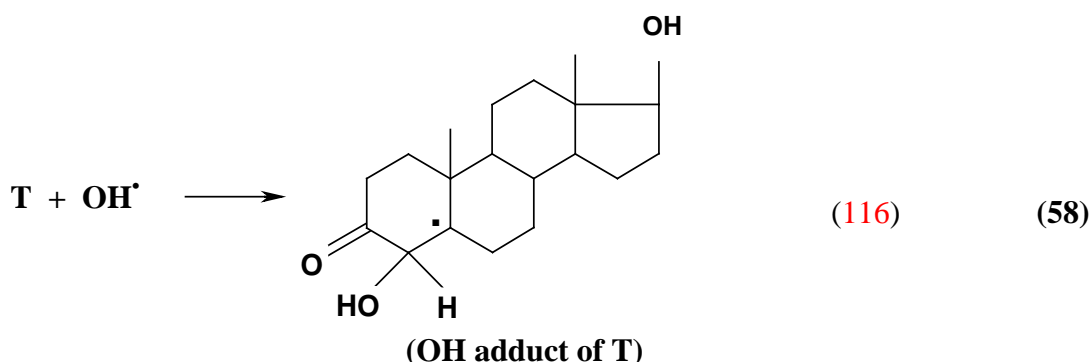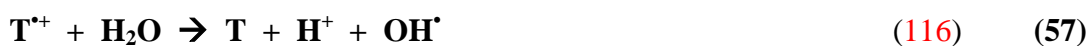
7.6.2. Regeneration of T by vitC

HPLC-analyses were performed, in order to investigate a possible regeneration mechanism of T by a potent electron donor, such as vitC, as it has been previously postulated, that a regeneration of hormones could be a possible approach to reduce the number of carcinogenic metabolites resulting from electron emission. Based on the HPLC-data, the degradation (%) of 1×10^{-4} mol/L T, 0.95×10^{-4} mol/L vitC and their mixture (1×10^{-4} mol/L T and 0.95×10^{-4} mol/L vitC) was calculated (116). The results are depicted in Figure 48 as a function of the absorbed UV dose (hν/L). T and vitC in mixture degrade both much slower, when they are UV-irradiated in mixture (curves B and D, respectively). Comparing the degradation of UV-irradiated T alone (curve A) and in mixture with vitC (curve B), a regenerative effect of vitC on T is obvious. Based on the

initial quantum yields (Q_i) of the corresponding remainders, the regeneration of T and the consumption of vitC in percent are calculated: Under the applied experimental conditions, T is regenerated by electron transfer from vitC to 58.6%. At the same time, 34% vitC are consumed.

The radical cation of T ($T^{\bullet+}$, eq. 51b), resulting of the electron emission from T, can be regenerated by vitC according to eq. 56. As $T^{\bullet+}$ is a strongly oxidizing species, it is able to react with water, as previously observed for β-car$^{\bullet+}$ (117), leading to the production of $OH^{\bullet}$ radicals (eq. 57), which in turn can react efficiently with T forming *e.g.* OH adducts (eq. 57). However, the importance of this reaction (eq. 58) is minimized, because of the presence of ethanol in the medium (eq. 38).

Figure 48. Remainder (%) of A: 1×10^{-4} mol/L T alone and B: in mixture with 0.95×10^{-4} mol/L vitC, C: 0.95×10^{-4} mol/L vitC alone and D: in mixture with 1×10^{-4} T dissolved in air-free solution of 40% water and 60% ethanol (pH ~7.4, 37°C), as a function of absorbed UV quanta (hv/L; λ=254 nm) (116).

$$\text{T}^{\bullet+} + \text{e}^-_{aq} \text{ (from vitC)} \rightarrow \text{T} \qquad\qquad (116) \qquad (56)$$

$$\text{T}^{\bullet+} + \text{H}_2\text{O} \rightarrow \text{T} + \text{H}^+ + \text{OH}^\bullet \qquad\qquad (116) \qquad (57)$$

$$\text{T} + \text{OH}^\bullet \longrightarrow \qquad\qquad\qquad (116) \qquad (58)$$

(OH adduct of T)

Transients like $\text{T}^{\bullet+}$ (eq. 51b) and OH adducts of T (eq. 58) can react with various species ($\text{R}^\bullet_x$) present in the medium, leading to the formation of metabolites. At the same time, T molecules in ground state are able to react with a part of the emitted e^-_{aq}, as already mentioned, resulting in the formation of T radical anions (cf. eq. 55b).

The OH group on position 17 of the T molecule may also participate in the regeneration process. As illustrated by eq. 51a, electron emission from the OH group of position 17 results in the formation of a T radical ($\text{T}^\bullet$). $\text{T}^\bullet$ is able to react with electrons, from *e.g.* vitC, and is, therefore, regenerated (eq. 59).

$$\text{T}^\bullet + \text{e}^-_{aq} \text{ (from vitC)} + \text{H}^+ \longrightarrow \qquad\qquad (116) \quad (59)$$

(T)
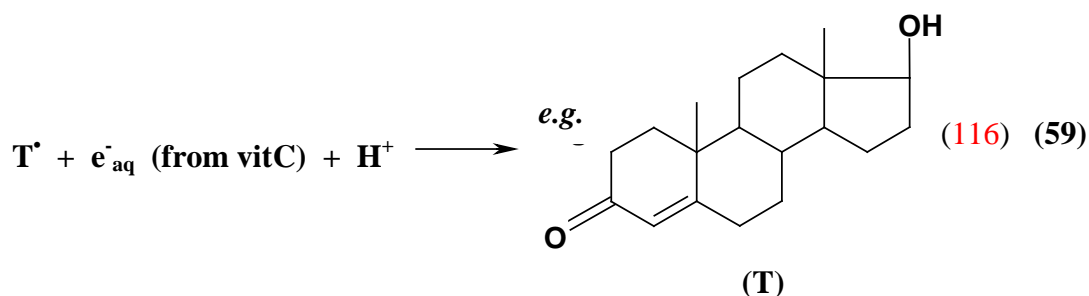
7.6.3. Effect of free radicals on T alone and in mixture with vitC

The effect of simultaneously generated oxidizing and reducing free radicals on the metabolites of T and the impact of vitC on the metabolite formation of T was simulated by experiments *in vitro*. As a model for living systems, the bacterial *Escherichia coli* strain AB1157 was implemented. The organism is producing the same types of free radicals.

As the media contained 4×10^{-2} mol/L ethanol, in aerated solutions only oxidizing ethanol peroxyl radicals operated. All investigated systems (5×10^{-5} mol/L T, 5×10^{-5} mol/L vitC and their mixture) showed slightly negative ΔD_{37} values, indicating, that the

metabolites resulting from the attack of ethanol peroxyl radicals exhibit an anti-proliferative effect on the bacteria. In media saturated with N_2O (ethanol radicals) and argon (ethanol radicals, e^-_{aq}), contrary results were obtained. The ΔD_{37} values of all susbtrates are positive, thus it can be stated, that T, vitC and their mixture show a protective effect under reducing experimental conditions.

Based on the molecular structure of T it is expected, that the ethanol peroxyl radicals ($^\bullet O_2C_2H_4OH$) predominantly attack the double bond of ring A of T, forming corresponding peroxyl adducts. Furthermore, to a smaller extent they are able to remove hydrogen from T. The resulting transients react with various species ($R^\bullet_x$) present in the medium, leading to the formation of metabolites with various biological properties. The reducing e^-_{aq} primarily react with the carbonyl group of ring A. Consequently, T radical anions (cf. eq. 55b) are produced, which give rise to various metabolites upon reaction with available species ($R^\bullet_x$) in the medium. The OH group on position 17 of ring D can also be involved in reactions with various types of species. However, in general, the corresponding reaction rate constants are rather low.

Summing up it can be stated, that oxidizing as well as reducing free radicals are both able to react very efficiently with T, resulting in the formation of a number of metabolites with varying biological properties. Hereby, vitC enhances the particular effect of T.


7.7. Water soluble hormones

7.7.1. Electron emission of water soluble hormones

E2 and P4 were obtained as water-soluble complex with HBC, as previously mentioned. Consequently, the obtained results from studies concerning the electron emission of wsE2 and wsP4 are an outcome of several, simultaneously proceeding reactions (113): firstly from ejection of e^-_{aq} of the specific hormone and of HBC upon UV-excitation, and secondly by the partly consumption of e^-_{aq} by the substrates, as the reaction rate constants of both hormones with electrons are rather high: $k(E_2+e^-_{aq})$ = $2.7x10^{10}$ L.mol^{-1}.s^{-1} (54), $k(P4+e^-_{aq})$ ~$4x10^9$ L.mol^{-1}.s^{-1} (99). The reaction rate constant of HBC and electrons is much lower: $k(HBC+e^-_{aq})$ = $8x10^7$ L.mol^{-1}.s^{-1} (92). Thirdly, the photolytic products can also participate in the mentioned processes, and therefore influence the yield of solvated electrons.
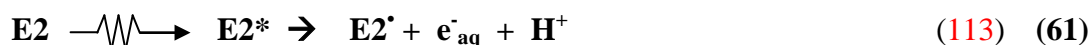
For the observed curve of electron emission of $1x10^{-4}$ mol/L HBC, which exhibits several maxima, indicating the involvement of multi-stage processes, it can be stated, that

with increasing absorbed UV quanta the pH of the medium declines (inset II, Figure 26). This decrease of pH indicates that the OH-groups of the HBC molecule are the primary source of electron ejection (eq. 60).

$$\textit{e.g.} \quad ROH \quad \longrightarrow \quad (ROH)^* \quad \longrightarrow \quad RO^\bullet + H^+ + e^-_{aq} \qquad (113) \quad (60)$$
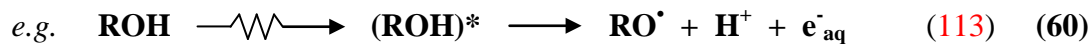
RO$^\bullet$ HBC radicals are generated, which are certainly involved in subsequent processes.

In experiments with wsE2 and wsP4 it was also found, that with increasing absorbed UV dose the pH of the media declined. Therefore, for E2 the phenolic ring A is assumed to be the source for the formation of H^+ (eq. 61). P4 results after UV-irradiation in a radical cation (P4$^{\bullet+}$, 47). P4$^{\bullet+}$ in turn is able to react with water, whereby it is regenerated to P4. By-products of this reaction (eq. 62) are OH$^\bullet$ and H^+, the latter of which is responsible for the observed pH decrease.

$$E2 \quad \longrightarrow \quad E2^* \rightarrow E2^\bullet + e^-_{aq} + H^+ \qquad\qquad\qquad (113) \quad (61)$$
$$P4 \quad \longrightarrow \quad P4^* \rightarrow e^-_{aq} + PRG^{\bullet+} + H_2O \rightarrow H^+ + PRG + OH^\bullet \quad (113) \quad (62)$$

The obtained $Q(e^-_{aq})$ values from $1x10^{-4}$ mol/L wsE2 ($Q(e^-_{aq}) = 7x10^{-3}$) and $1x10^{-4}$ mol/L wsP4 ($Q(e^-_{aq}) = 4x10^{-4}$) differ by one order of magnitude (insets, Figure 27). Previously determined $Q(e^-_{aq})$ values for $1x10^{-4}$ mol/L E2 and $1x10^{-4}$ mol/L P4 in the same solvent mixture, but dissolved in aerated medium, also show a difference, which is even bigger: $Q(e^-_{aq}) = 9.1x10^{-3}$ for E2 and $Q(e^-_{aq}) = 3.45x10^{-4}$ for P4 (47). However, the difference of the obtained quantum yields of solvatzed electrons was explained by the differences in the molecular structures of the two hormones: As mentioned before, E2 emits electrons predominantly from the OH group of the aromatic ring A. Furthermore, there is another OH group at position 17 of ring D, which also contributes to the emission of electrons. P4, in contrast, has no OH groups at all. Only the π-electron structure at ring A (double bond at positions 4–5) is favouring the emission of electrons. However, two carbonyl groups, one located at ring A and one at ring D, consume electrons with a rather high reaction rate constant of $k(CO+e^-_{aq}) = 1.7x10^9$ L.mol^{-1}.s^{-1} (92). This fact also explains the low $Q(e^-_{aq})$ values observed for the mixture of wsE2 and wsP4 (Figure 28).

7.7.2. Effect of free radicals on water-soluble hormones

In experiments *in vitro* using *Escherichia coli* bacteria as model, the effect of oxidizing ($OH^\bullet$, $O_2^{\bullet-}$, *etc.*) and reducing (e^-_{aq}, $H^\bullet$, $R^\bullet$, *etc.*) free radicals, which are also produced by the organism, on the metabolite formation of wsE2, wsP4 and their mixture was investigated (114). Comparing the ΔD_{37} values of HBC and wsE2 obtained in media saturated with air (Figure 36) or N_2O (Figure 37), it can be stated, that all are positive, indicating that the resulting metabolites exhibit a proliferative effect on the cells. The ΔD_{37} value of wsE2 is more than 6 times higher in medium saturated with N_2O. As 90% $OH^\bullet$ radicals operate under these experimental conditions, compared to 46% in aerated medium, the effect is probably due to a very high scavenging ability of the resulting metabolites for $OH^\bullet$. Since in the experiments in air-free media negative ΔD_{37} values were observed, the reducing free radicals (44% e^-_{aq}, 10% $H^\bullet$) seem to lead to the formation of metabolites with cytostatic properties.

$OH^\bullet$ radicals attack preferentially the phenolic ring A of E2, due to the somewhat higher reaction rate constant of $OH^\bullet$ with phenol ($k=1\times10^{10}$ $L.mol^{-1}.s^{-1}$) than with *e.g.* cyclohexane ($k=6.1\times10^{9}$ $L.mol^{-1}.s^{-1}$). This illustrated by eq. 63. After splitting off H_2O from the resulting OH adduct (eqs. 63,64), an E2 phenoxyl radical is formed. $OH^\bullet$ radicals are able to abstract H atoms from other rings, as well, but with somewhat lower k values.

(63)
(114)

**(E2)**   **(OH adduct of E2)**   **(E2 phenoxyl radical)**
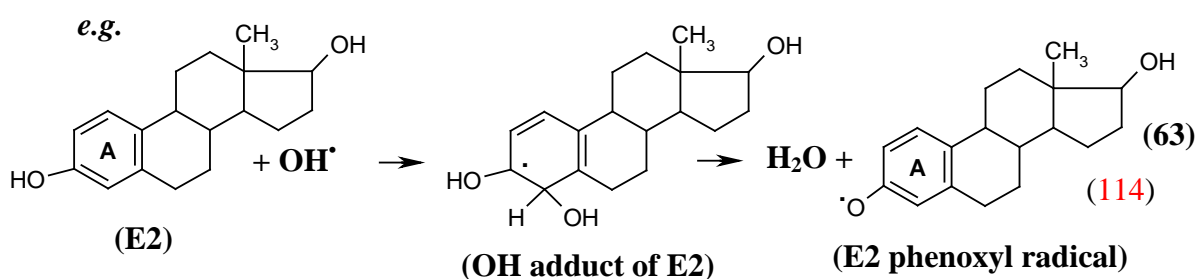
$OH^\bullet$ can be added to ring A of E2 on ortho, meta, para or ipso position. This is shown for ortho position in eq. 64, taking phenol as example for ring A (65).

e.g. H_2O +   *(ca. 70%)*   (114)   **(64)**

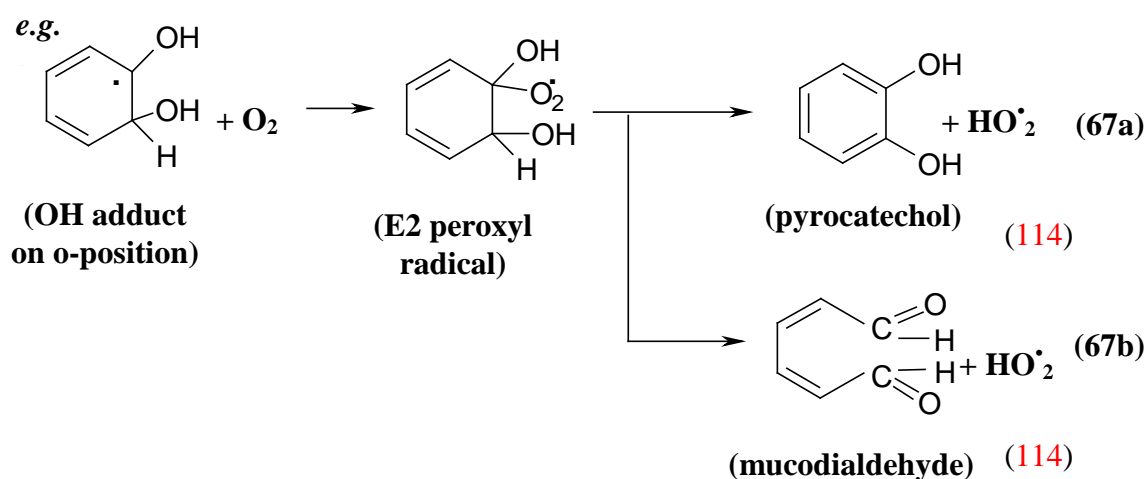**(OH adduct on o-position)**   **(E2 phenoxyl radical)**
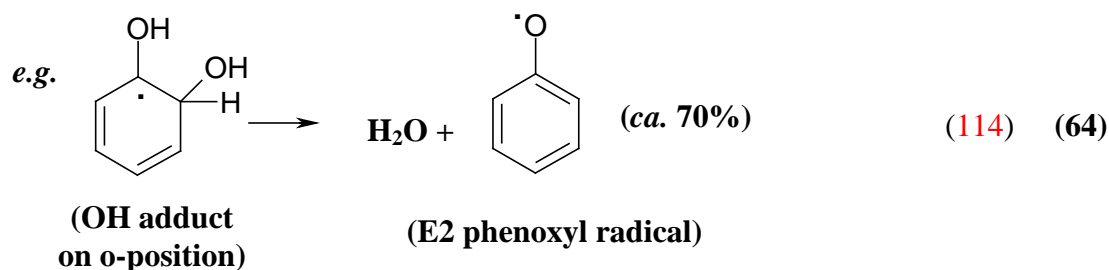
Taking again phenol as example for ring A of E2, the following mesomere forms of phenoxyl radicals are possible (eq. 65), each of which can react with other compounds available in the medium, leading to the formation of countless metabolites with different biological properties.
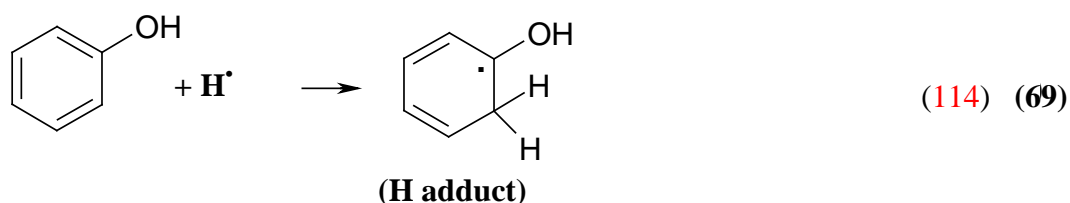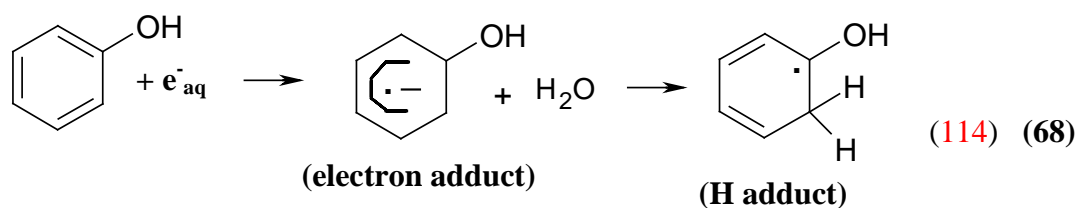

(114) **(65)**
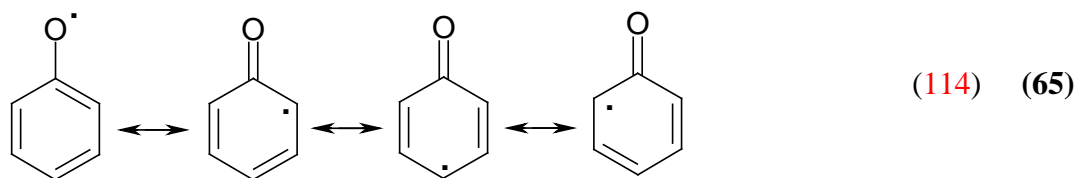
OH adducts are also able to undergo disproportion (eq. 66) instead of forming a phenoxyl radical type after splitting off water. In doing so, E2 is regenerated and pyrocatechol and H_2O are formed as by-products.

2 C₆H₅OH(OH) → **C₆H₅OH +** ... **+ H₂O**

(OH adduct on o-position)

(pyrocatechol)

(114) **(66)**
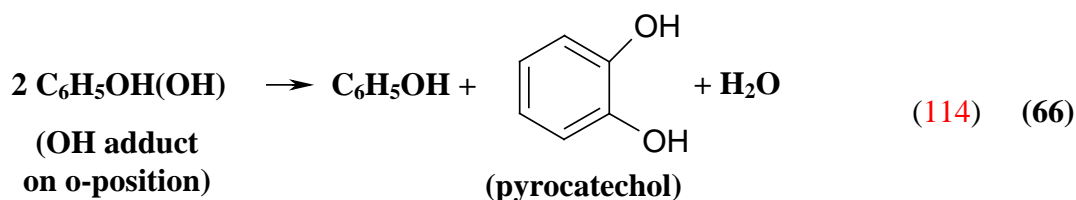
Another possible reaction mechanism of OH-adducts is the addition of oxygen, when available, resulting in the formation of E2 peroxyl radicals. These peroxyl radicals subsequently decompose to various products and superoxide radicals (eq. 67a,67b):

(OH adduct on o-position)

(E2 peroxyl radical)

(pyrocatechol) (114)

+ HO˙₂ **(67a)**

+ HO˙₂ **(67b)**

(mucodialdehyde) (114)

However, based on the molecular structure of wsE2, it is expected, that e⁻$_{aq}$ and H˙ will predominantly attack the A-ring and to a smaller extent other positions of the molecule. Taking again phenol as a model for ring A of wsE2 the following reactions are possible:

$$\text{(phenol)} + e^-_{aq} \longrightarrow \text{(electron adduct)} + H_2O \longrightarrow \text{(H adduct)} \qquad (114) \quad (68)$$

(electron adduct)   **(H adduct)**

$$\text{(phenol)} + H^\bullet \longrightarrow \text{(H adduct)} \qquad (114) \quad (69)$$

(H adduct)

As already discussed for the addition of $OH^\bullet$ radicals on ring A of E2, e^-_{aq} and $H^\bullet$ can add on various positions (ortho, para, meta or ipso) of ring A with a very high reaction rate constant of $k(e^-_{aq}+E2) = 2.7 \times 10^{10}$ L.mol^{-1}.s^{-1} (54). Further, the resulting H adducts are also able to react with intermediates or other compounds, present in the medium, forming various kinds of metabolites.

In general it can be stated, that E2 reacts very efficiently with all kinds of free radicals, produced as primary species of water radiolysis. These reactions give rise to countless, subsequent reactions, in the course of which transients may react with transients or other compounds present in the medium. A broad spectrum of metabolites with different biological properties results from this bulk of reactions.

In the experiments *in vitro* with the bacterial *Escherichia coli* strain AB1157 treated with wsP4 alone and in mixture with wsE2 prior to γ-irradiation, it was surprisingly found that the ΔD_{37} values obtained in aerated medium and medium saturated with N_2O are contrary: In aerated medium, the ΔD_{37} values of 1×10^{-4} mol/L wsP4 and the mixture of 1×10^{-4} mol/L wsP4 and 1×10^{-4} mol/L wsE2 are negative, indicating a cytostatic effect of the resulting metabolites (Figure 40), whereas in medium saturated with N_2O the corresponding ΔD_{37} values are positive (Figure 41). Under both experimental condition, oxidizing radicals are the predominant species, but in the latter case, 10% of the radicals are $H^\bullet$, a reducing species, which may contribute to the observed effect. Considering the ΔD_{37} values obtained in media saturated with argon (Figure 42), where the fraction of the reducing species is 54% (10% $H^\bullet$, 44% e^-_{aq}), which match very well with those found in medium saturated with N_2O, the determining role of the reducing species is obvious in this respect.

Based on the molecular structure of P4 it is expected, that the oxidizing radicals add predominantly to the π-electron structure of ring A. In contrast, the reducing species primarily react with the carbonyl groups of ring A and D. The resulting transients of all these processes may regenerate, react with each other or with available species ($R^{\bullet}_x$) in the medium. Hence, as already explained for T and E2, the formation of various metabolites is conceivable, each of which having unique biological properties.

Comparing the ΔD_{37} values of wsE2 and wsP4, it can be stated, that the two hormones show an opposite behaviour in aerated as well as in air-free media, pointing out the contrary biological action of wsE_2 and wsP4. In the mixture, wsE2 enhances the particular effect of wsP4. Therefore, mixtures of these hormones could be implemented for reduction of the number of carcinogenic metabolites. However, it should be noted, that both hormones are influenced by HBC.

8. CONCLUSION

In the present study it was shown, that the sex hormones T (98,99), wsE2, wsP4 (113) and phytoestrogen GEN (112) are able to emit electrons upon excitation to their singlet state in polar media. This was simulated by excitation with monochromatic UV-light (λ=254 nm; 4.85 eV/hν). Hereby, it was shown, that the yield of the resulting solvated electrons (e^-_{aq}) depends mainly on the specific molecular structure of the substrate, concentration and temperature. In addition, the absence or presence of oxygen during the dissolving process of hormones was found to be of importance, as much higher quantum yields, $Q(e^-_{aq})$, calculated from the tangent of the corresponding curves, were obtained, when the hormones were dissolved in air-free media. This might be explained by a possible oxidation of the hormones when dissolved in the presence of oxygen, as oxygen occurs in triplet state in the atmosphere and is, therefore, rather reactive. The impact of the temperature on $Q(e^-_{aq})$ values, which increase with temperature, might be due to the fact that with rising temperature the emission of e^-_{aq} results from a higher vibrational level of the singlet state.

Interestingly, the molar extinction coefficients at λ=254 nm (ε_{254} values), calculated from absorption spectra with the help of the Lambert-Beer-Law, decreased with increasing substrate concentration (98,99,112,113). This observation indicates the formation of hormone associates (hormone clusters), which are preferentially built with higher substrate concentrations. Furthermore, it was found that with increasing substrate concentration (mol/L) the $Q(e^-_{aq})$ values decreased accordingly. This can be due to the formation of associates, as the hormone molecules in ground state within a hormone cluster may consume ejected electrons, resulting in an increased "self-consumption" of e^-_{aq}. Moreover, the dissimination of the absorbed UV quanta might not occur homogenously within the hormone associates, causing energy quenching. Photolytic products, which are formed as a result of electron emission from hormones, can also consume emitted electrons. The latter is illustrated by the wave-like course of the curves of electron emission, exhibiting several maxima, which usually decrease with ongoing UV-irradiation. By HPLC analyses it was shown that the hormones were decomposed in large part after absorption of relatively low UV doses. Comparing these results with the obtained curves of electron emission, it can be stated that only the first peak belongs exclusively to the electron emission from the particular hormone itself. Observed further

peaks are strongly influenced by the electron emission ability of generated photolytic products.

As already mentioned, the molecular structure of a certain compound is the crucial factor for its ability to emit electrons. As the $Q(e^-_{aq})$ value observed for GEN is lower than that of E2, which has been previously established (47), it can be stated, that the carbonyl group (C=O) of GEN as well as the oxygen in the middle ring are decisive in this respect. Both consume electrons with rather high reaction rate constants of $k(CO+e^-_{aq}) = 1.7 \times 10^9$ $L.mol^{-1}.s^{-1}$ and $k(O_2+e^-_{aq}) = 1.9 \times 10^{10}$ $L.mol^{-1}.s^{-1}$, respectively (92,100). E2 in contrast mainly emits electrons, predominantly from the OH group of the aromatic ring A. Comparing T, P4 and E2, E2 turns out to have the highest $Q(e^-_{aq})$ value, followed by T and finally P4. This fact is due to the different molecular structures of the hormones at ring A and D: Ring A of E2 is phenolic, as already mentioned, favouring the electron emission, whereas ring A of T and P4 only has a double bond on position 4-5, which is a source of electron emission, indeed, but not as efficient. Furthermore, a carbonyl group is situated on ring A of T and P4, which consumes a part of the emitted electrons, explaining the higher observed $Q(e^-_{aq})$ value for E2. Ring D of both E2 and T carries a hydroxyl group on position 17, which again favours the emission of electrons. P4, in contrast, has a further carbonyl group on this position. Therefore, P4 emits the lowest yields of solvated electrons of these hormones.

The established ability of sex hormones to both emit and consume electrons classifies them as "electron mediators". This behaviour makes a communication by e^-_{aq} between different biological systems in the organism conceivable (118).

Using vitC as representative for potent electron donors, it was further shown by HPLC that transients resulting from electron emission of T can be partly regenerated to their original structure by electron transfer processes, if they are still in "status nascendi". Due to the regeneration of T less metabolites are formed. Consequently, also the number of metabolites initiating neoplastic processes is assumed to be reduced.

As the organism permanently generates oxidizing (OH, $O_2^\bullet$, *etc.*) and reducing (e^-_{aq}, $R^\bullet$, *etc.*) free radicals, which are crucial for various biological processes, it was of interest to investigate the reactions of these radicals with various hormones in the frame of experiments *in vitro* with *Escherichia coli* bacteria (AB1157) in aqueous media (pH ~7.4). By application of appropriate experimental conditions (saturation of the solutions with various gases), desired kind and concentration of primary free radicals were obtained

as a result of the radiolysis of water. WsE2 (embedded in HBC) was found to be a very efficient radical scavenger of oxidizing radicals, which attack the hormone mainly on its phenolic ring A. As a consequence, several mesomere forms of transients are possible, which lead to the formation of metabolites with predominant proliferative effects. The attack of reducing species (e^-_{aq}, $H^\bullet$) occurs to a large part at ring A, as well. However, the resulting metabolites show strong anti-proliferative properties. WsP4 with incorporated HBC, in contrast, shows an opposite behaviour. The reducing species, which play a very important role for its biological effect, are expected to primarily react with the carbonyl groups of ring A and D, resulting in the formation of radical anions. Mixtures of wsE2 and wsP4 intensify the effects observed for wsP4, indicating that a combination of wsP4 and wsE2 may strongly reduce the number of carcinogenic metabolites of wsE2.

T and a mixture of T and vitC in a media containing 4×10^{-2} mol/L ethanol were also investigated in this respect. T intermediates resulting from attack of oxidizing free radicals on the double bond of ring A show weak cytotoxic properties. Under reducing conditions, T induces strong proliferation of *E.coli* bacteria. These effects are both enhanced, when T is combined with vitC.

Concluding it might be said, that all investigated sex hormones readily react with various oxidizing and reducing free radicals, according to their specific molecular structures with various functional groups and double bonds. The resulting transients, which often have several mesomere structures, are very eager to react with other substances. The biological action of the investigated hormones and their mixtures, therefore, depends on the available compounds in the media. These can critically influence the behaviour of the resulting metabolites towards cells.

The present research work represents an attempt to get a deep insight in the enormously complicated, but at the same time synchronized and overlapping biological processes, which involve hormones and their metabolites. Though some promising results were obtained, much more work is needed in order to draw these secrets from nature!

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10. CURRICULUM VITAE

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PUBLICATIONS

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