Formation of Sex Hormone Transients Resulting from Attack of Free Radicals

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Abstract. *Background: Transients of the sex hormones testosterone (TES) and estrone (E1) exhibit an impact on the carcinogenesis of most prostate and breast cancer types. For elucidation of involved reaction mechanisms, in vitro, experiments using γ-ray for generation of attacking hormone transients and UV-light (λ=254 nm) for excitation of hormone molecules were applied. Materials and Methods. Experiments in vitro (Escherichia coli AB1157) incubated with TES and E1, individually as well as in mixture with vitamin C (electron donor), were performed under γ-irradiation in water-alcohol (40/60) medium for clarifying-up the reaction mechanism. The hormone degradation/regeneration processes were studied by high performance liquid chromatography analysis. Results: Independently of hormone molecular structure, the determining factor for the biological properties, such as carcinogenity, were found to be based on the hormone transients. The biological ability of these, however, depends on the chemical properties of the species attacking the corresponding hormone. Hormone degradation can be, at least partly, converted into hormone regeneration by electron transfer from an electron donor (e.g. vitamin C), when available during the period of status nascendi of the hormone radicals.*

It has been recently shown that hormones are able to eject solvated electrons (e_{aq}^-), but can also react with e_{aq}^- as well as transfer them to other biological systems in an organism (1-6). Since each hormone emits e_{aq}^- with a specific frequency, a new communication pathway between biological systems by electron transfer processes *via* the brain was hypothesized (7). e_{aq}^- Represent the basic form of the H-atom, which is the reducing acid species in aqueous media [see *e.g.* (8)]. The existence of e_{aq}^- was established for the first time by the transformation of aqueous CO_2 into simple organic compounds under irradiation with γ-ray, as well as by UV-light (9, 10). Subsequently, the absorption spectrum of e_{aq}^- in water was determined by pulse radiolysis (λ=720 nm) (11), as well as of trapped electrons (e_{aq}^-) in ice-matrix at 77°K (λ=575 nm) (12). It was subsequently found that riboflavin (vitamin B2) as well as a number of its derivatives in water-methanol media can also emit e_{aq}^- under UV irradiation (λ=254 nm) (13). The ability to eject e_{aq}^- has also been established to be valid for antioxidant vitamins, as well as for a number of B-series vitamins (14). Several studies showed that the yield of emitted e_{aq}^- depends on the molecular structure of the compounds, excitation energy, substituents (*e.g.* –OH, $-OCH_3$, $-OPO_3H_2$, -COO-, $-NH_2$, $-NHCH_3$), temperature and pH of the aqueous solution (15). It was also experimentally shown that hormones can be regenerated within the lifetime (*status nascendi*) of their transients by electron transfer from a potent electron donor, such as vitamin C (16, 17), as well as by mutual action of two hormones, one of which acts as an electron donor (18).

Enzymes act as catalysts and energy providers in living cells, as well as by enabling the chemical metabolism of compounds by oxidizing free radicals (OH, $O_2^{\bullet-}$, $^{\bullet}RO_2$) generated by oxidase or reducing species (H, e_{aq}^-, $^{\bullet}R$) delivered by reductase (19, 20). These free radicals can also be generated by treatment of aqueous solution with radiation (γ-ray, electrons, UV-light *etc.*). Following this pathway, it was possible to investigate the effect and the reaction mechanisms of the attack of free radicals on hormones in *Escherichia coli* AB1157 (21, 22), as well as breast cancer cells (4, 23). The survival curves of *Escherichia coli* obtained from 17β-estradiol (17βE2), embedded in water-soluble cyclodextrin (HBC) and exposed to, γ-ray in various environments, showed that 17βE2 and HBC act as very efficient scavengers for

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oxidizing radicals (OH, $O_2^{\bullet-}$). However, the 17βE2 transients generated by the attack of reducing free radicals (e_{aq}^- , H) exhibit a strong anticancer property (5). Similar experiments *in vitro*, using 17βE2 and progesterone (PRG) embedded in HBC, exhibit opposite effects (22). Namely, in aerated aqueous media (acting radicals: OH and $O_2^{\bullet-}$), both hormones have a strongly pronounced cytostatic property. However, the action of reducing primary radicals (e_{aq}^-, H) on the hormones leads to a radical-scavenging effect. In experiments *in vitro* applying PRG and 17α-hydroxyprogesterone (17αOHPRG) in aqueous media containing 4×10^{-2} mol.l^{-1} ethanol (hormone solubility mediator), the primary radicals (H, OH, $O_2^{\bullet-}$) are converted into the corresponding ethanol transients ($^{\bullet}C_2H_4OH$, $^{\bullet}O_2C_2H_4OH$) (4). Therefore, only the e_{aq}^- and the ethanol radicals attack the hormones. The generated hormone transients of PRG and 17α-OHPRG have a rather high cytostatic effect. Furthermore, experiments *in vitro* (MCF-7 breast cancer cells) in the presence of estrone (E1), PRG and their combination, which were γ-irradiated in media containing 2×10^{-4} mol.l^{-1} ethanol, also had a strong cytostatic effect, especially using a combination of both hormones (23). Hence, in this case, the biological effect is caused by e_{aq}^- and the ethanol radicals. The action of genistein (GEN) transients, generated by the attack of oxidizing and reducing free radicals was studied in various media using MCF-7 cells (24). It was found that GEN transients sensitize the used strain against the action of oxidizing (OH, $O_2^{\bullet-}$) and reducing (e_{aq}^-, H) free radicals. GEN transients also enhanced the action of mitomycin C (MMC) in both oxidizing and reducing environments.

From the present brief overview of the experiments carried out *in vitro* it is clear that the transients generated by free radical attack on hormones have a different cytostatic action from the parent hormone. Obviously, the complexity of the involved reaction mechanisms of hormones depends on several factors, such as hormone molecular structure, environment, type of attacking radical, reaction rate constants and mutual interaction of hormone free radicals. Hence, in order to gain a better understanding of the factors leading to hormone-induced cancer, further experiments *in vitro* were performed using testosterone (TES) and E1 as representative hormones of different molecular structures. Using vitamin C as an electron donor and the hormones as acceptors, high performance liquid chromatography (HPLC) analyses were also performed in order to study the degradation of the substances under UV-irradiation and the possible regeneration of the hormones in the presence of vitamin C as an electron donor.

Materials and Methods

Chemicals. All used chemicals were of highest purity available (Fluka, Aldrich, Merck) and were used as obtained. Triple distilled water, containing 4×10^{-2} mol•l^{-1} ethanol (solubility mediator for the hormones; media pH~7.4) was used for preparation of the hormone solutions. In order to achieve the desired type of primary free radicals, the solutions were saturated with high purity air, N_2O or argon, respectively, prior to irradiation.

Radiation. A Gammacell 220 facility (Nordion International Inc., Kanata, Canada) providing a dose rate of 30 Gy/min served as irradiation source for the production of primary free radicals originating from the solvent. Additionally, monochromatic UV-light (λ=254 nm; E=4.85 eV hν^{-1}) provided by a low-pressure Hg-lamp (HNS 12, Osram 12 W) with incorporated Vycor-filter for removal of the 185 nm line, was used. The UV-equipment and actinometry were described previously (16, 17, 22).

Analysis. HPLC analyses were performed in order to follow the temporal concentration change of the used substrates under irradiation. The HPLC facility: Hewlett-Packard Agilent 1100 HPLC-series model 1046 with computer online was also reported previously (4, 17). Samples of 25 μl were injected and separated by a linear elution gradient between the mobile phases 2.5×10^{-4} mol, l^{-1} ammonium acetate in water (A) and acetonitrile (B). The gradient started with 80% A, decreased linearly to 65% A in 0.6 min, followed by a linear change to 10% in 23.4 min, was then held at 10% A for 3 min, increased afterwards to 80% A within 3 min and finally held at 80% A for 5 min with a flow of 0.25 ml.min^{-1}.

Experiments in vitro. The handling of *Escherichia coli* AB1157 (DSMZ, Braunschweig, Germany) used as a model for living systems was described previously (21-23). The change of the bacterial survival ratio N/N_0 (N_0=number of colonies before and N = number of colonies after irradiation) was studied as a function of the absorbed radiation dose (Gy); 1 Gy=6.24×10^{15} eV•ml^{-1}) in a dose range up to 450 Gy. Thereby the media contained: (i) 5×10^{-5} mol•l^{-1} TES, (ii) 5×10^{-5} mol•l^{-1} vitamin C and (iii) TES plus vitamin C each at 5×10^{-5} mol•l^{-1}. In all bacterial solutions 4×10^{-2} mol•l^{-1} ethanol was used. Similar experimental series were also performed with E1, vitamin C and their combination.

Involved reactions. For a better understanding of the subject matter some fundamentals about water radiolysis are firstly mentioned. Using γ-rays water is radiolysed to a number of primary products, the yields of which (G-value=number of species formed by absorption of 100 eV energy) are given in brackets in cross reaction (Eqtn 1).

H_2O ---ΛΛΛΛ→ e_{aq}^- , H, OH, H_2, H_2O_2, Haq^+, OH_{aq}^-
(G-values, pH 6-8)
(2.7) (0.6) (2.8) (0.45) (0.72) (3.2) (0.5) (Eqtn 1)

Depending on the gas (argon, N_2O, air) used for saturation of the aqueous media and considering the corresponding reaction rate constants, the composition of the primary acting free radicals is changed as given in the insert of the corresponding Figure.

Since all solutions contain 4×10^{-2} mol l^{-1} ethanol the following competitive reactions also took place:

$H + C_2H_5OH \rightarrow H_2 + {}^{\bullet}C_2H_4OH$,
$k=1.7\times10^7$ l•mol^{-1}•s^{-1} (25) (Eqtn 2)

$OH + C_2H_5OH \rightarrow H_2O + {}^{\bullet}C_2H_4OH$,
$k=1.9\times10^9$ l•mol^{-1}•s^{-1} (25) (Eqtn 3)

The solvated electrons (e_{aq}^-) practically do not react with alcohol. Therefore, in airfree medium, the acting radicals are e_{aq}^-, H and OH as well as $^{\bullet}C_2H_4OH$.

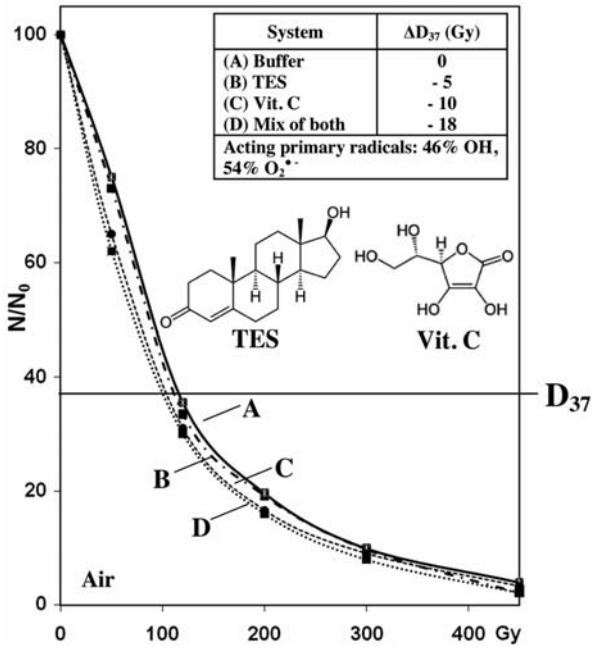

Figure 1. *Survival curves (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous aerated media containing 4×10^{-2} mol•l^{-1} ethanol (pH≅7.4) in the presence of: buffer (A), 5×10^{-5} mol•l^{-1} TES (B), 5×10^{-5} mol•l^{-1} vitamin C (C) and mix of 5×10^{-5} TES and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

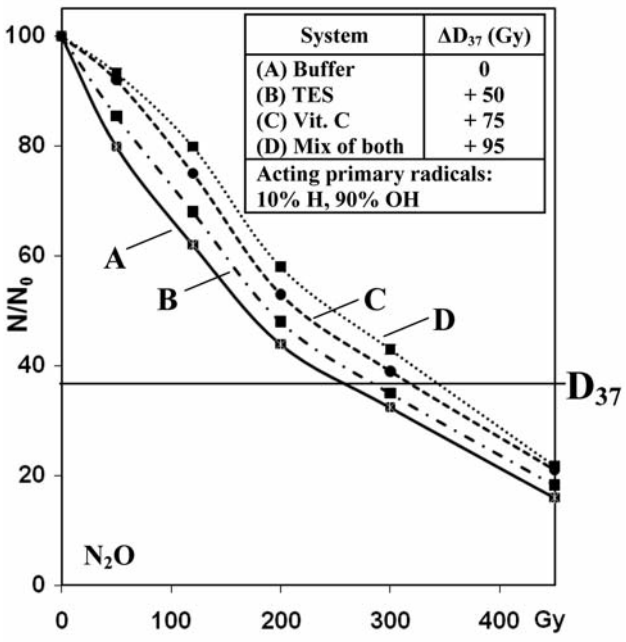

Figure 2. *Survival curves (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous media saturated with N2O containing 4×10^{-2} mol•l^{-1} ethanol (pH≅7.4) in the presence of: buffer (A), 5×10^{-5} mol•l^{-1} TES (B), 5×10^{-5} mol•l^{-1} vitamin C (C) and mix of 5×10^{-5} mol•l^{-1} TES and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

In solutions saturated with N_2O, the e_{aq}^- are converted into OH radicals:

$e_{aq}^- + N_2O \rightarrow OH + OH^- + N_2$,
$k=0.91\times10^{10}$ l•mol^{-1}•s^{-1} (25) (Eqtn 4)

Hence, H, OH and $^\bullet C_2H_4OH$ transients are the reacting species with the respective substrate in the medium.

Finally, in aerated medium, the H atoms and e_{aq}^- are converted into peroxyl free radicals, which subsequently react with ethanol in medium (pH~7.4) forming ethanol peroxide (Eqtn 5-10):

$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-}$, $k=1.9\times10^{10}$ l•mol^{-1}•s^{-1} (25) (Eqtn 5)
$H + O_2 \rightarrow HO_2^\bullet$, $k=2.0\times10^{10}$ l•mol^{-1}•s^{-1} (25) (Eqtn 6)
$HO_2^\bullet \leftrightarrow H+ + O_2^{\bullet-}$, pK=4.8/26/ (Eqtn 7)
$O_2^{\bullet-} + C_2H_5OH \rightarrow {}^\bullet C_2H_4OH + HO_2^-$ (ethanol radical) (Eqtn 8)
$O_2^{\bullet-} + {}^\bullet C_2H_4OH \rightarrow {}^-O_2C_2H_4OH$ (ethanol peroxide) (Eqtn 9)
$O_2 + {}^\bullet C_2H_4OH \rightarrow {}^\bullet O_2C_2H_4OH$ (ethanol peroxide radical) (Eqtn 10)

Therefore, in aqueous media saturated with air, the $O_2^{\bullet-}$ pecies and the unstable peroxides ($^\bullet O_2C_2H_4OH$ and $^-O_2C_2H_4OH$) interact with the other components.

Results

TES/vitamin C system: Survival curves. The change of the bacterial survival ratio N/N0 was first studied in aerated solutions containing 5×10^{-5} mol•l^{-1} TES, 5×10^{-5} mol•l^{-1} vitamin C as well as in a mixture of both, using the same concentrations. As already mentioned all solutions contained 4×10^{-2} mol.l^{-1} ethanol as hormone solubility mediator. Figure 1 presents the survival curves of the bacteria: N/N0 ratio as a function of the absorbed radiation dose (Gy; 1 Gy = 6.24×10^{15} eV•g^{-1} in aerated media). The curves are very close to each other. The TES intermediates, resulting from the above mentioned oxidizing agents, have cytostatic properties. Bacteria incubated with vitamin C as well as with the mixture of TES and vitamin C have also a cytostatic effect, whereby the mixture exhibits a more pronounced impact in respect to radiation sensitization. This is expressed by the ΔD_{37}(Gy) values, derived from the corresponding survival curves and given as an insert in Figure 1. The ΔD_{37}(Gy) represent values at a radiation dose (Gy) at which N/N_0=0.37. Hence, ΔD_{37} (Gy)=D_{37} (Gy) sample – D_{37} (Gy) buffer. Obviously, in media saturated with air the $O_2^{\bullet-}$ and OH species as well as mainly the ethanol transients resulting from reaction in Eqtn 8 to Eqtn 10 interact with the included substrates.

Similar experiments *in vitro* in media saturated with N_2O were conducted and the obtained survival curves are shown in Figure 2. According to reactions shown in Eqtn 2 to Eqtn 4, in addition to H and OH, $^\bullet C_2H_4OH$ transients were also involved in the reactions with the substrates. The calculated ΔD_{37}(Gy) values are presented as an insert in Figure 2, and demonstrate exclusively a protective effect under γ-irradiation.

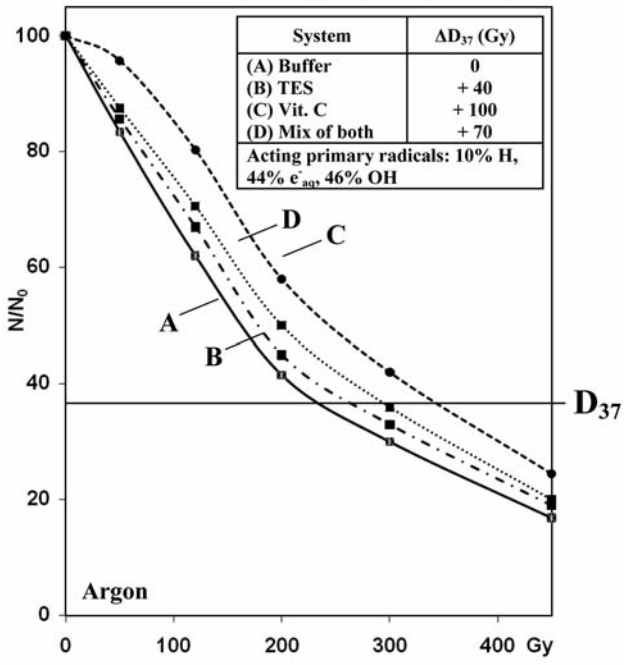

Figure 3. *Survival curves (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous media saturated with argon, containing 4×10^{-2} mol•l^{-1} ethanol (pH≅7.4) in the presence of: buffer (A), 5×10^{-5} mol•l^{-1} TES (B), 5×10^{-5} mol•l^{-1} vitamin C (C) and mix of 5×10^{-5} mol•l^{-1} TES and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

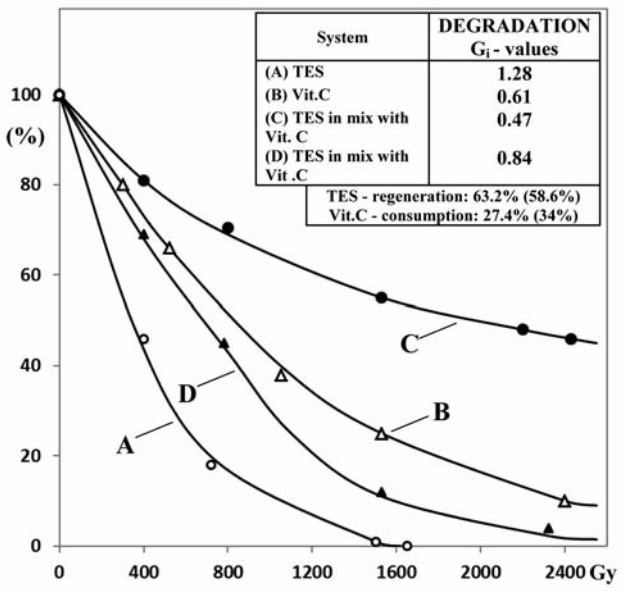

Figure 4. *High Performance Liquid Chromatography analyses. Degradation of: 10^{-4} mol•l^{-1} TES (A), 1×10^{-4} mol•l^{-1} vitamin C (B), 1×10^{-4} mol•l^{-1} TES in the presence of 1×10^{-4} mol•l^{-1} vitamin C (C) and 1×10^{-4} mol•l^{-1} vitamin C in the presence of 1×10^{-4} mol•l^{-1} TES (D), as a function of absorbed UV-dose, converted into (Gy) units. The substrates were dissolved and UV irradiated in an airfree mix of 40% water + 60% ethanol (pH≅7.4, 37˚C). Insert. Initial degradation Gi-values, derived from the curves (A) to (D). TES regeneration and vitamin C consumption of previous results are given in brackets.*

Finally, in Figure 3 the survival curves of bacteria in medium saturated with argon are presented, which result by attack partly of the primary acting radicals (see Eqtn 1) and mainly by $^{\bullet}C_2H_4OH$ species the derived ΔD_{37}(Gy)-values are given as an insert. In this case H and OH as well as $^{\bullet}C_2H_4OH$ transients (Eqtn 2 and 3) together with e_{aq}^- are involved in reactions with the substrate present in the medium. The calculated ΔD_{37}(Gy) values are positive, indicating again a protective radiation effect of the system. However, it is interesting to note that the mix of both TES and vitamin C did not exhibit the expected most pronounced effect.

TES Regeneration: HPLC analysis. HPLC data resulting from 0.95×10^{-4} mol•l^{-1} TES and 1×10^{-4} mol•l^{-1} vitamin C, dissolved in a mix of 40% water and 60% ethanol (pH~7.4; 37˚C) and treated with UV-irradiation (λ=254 nm), individually and as a mixture of both, have already been reported (17). However, for a better comparison to the present data, the HPLC experiments were repeated using the same conditions. Thereby the applied UV dose was converted into Gy units taking E=4.85 eV•hv^{-1} and 1 Gy=6.24×10^{15} eV•ml^{-1} for the UV quanta. Substrate degradation shown as a function of absorbed dose are presented in Figure 4.

Obviously, vitamin C being a potent electron donor, it is able to regenerate the main part of TES transients by electron transfer, as long as they are still in *statu nascendi* (17). This effect is illustrated by the course of curve A (TES only) and that of curve C (mix of TES plus vitamin C) in Figure 4. This is in accordance with the photolysis of vitamin C (curve B) and its increased degradation in the presence of TES (curve D/Figure 4). The observed regeneration of TES and the consumption of vitamin C (see insert Figure 4) is in fair agreement with previous results mentioned above (see Figure 4, insert).

Estrone/vitamin C system: survival curves. Experiments *in vitro* (*Escherichia coli*, AB 1157) were also conducted with E1 individually and in combination with vitamin C. E1 was used as a representative of hormones having phenolic ring A in the molecule. The obtained survival curves using 10^{-5} mol•l^{-1} E1 (curve B) and 10^{-5} mol•l^{-1} Vit C (curve C) individually and a mixture of both (curve D) in aerated media (40% water + 60% ethanol) are presented in Figure 5. It is interesting to note that the derived ΔD_{37}(Gy) value of E1 (see insert Figure 5) is positive, indicating a distinct proliferative effect of the bacteria. This is in accordance

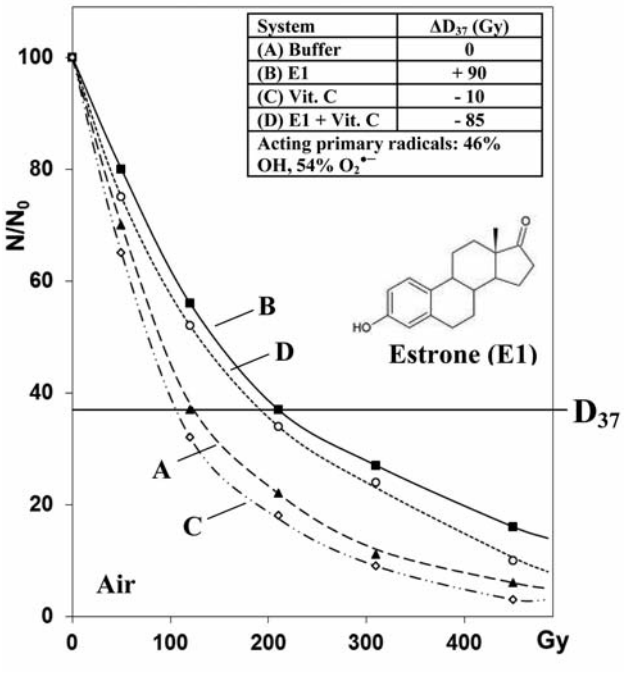

Figure 5. *Survival curves: (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous aerated media containing 4×10–2 mol•l–1 ethanol (pH≅7.4) in the presence of: buffer (A), 1×10–5 mol•l–1 E1 (B), 1×10–5 mol•l–1 vitamin C (C) and mix of respective 1×10–5 mol•l–1 E1 and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

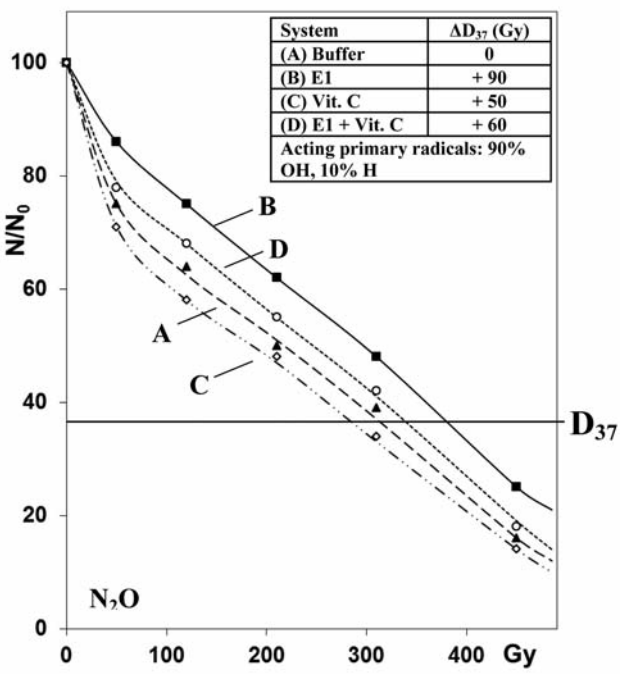

Figure 6. *Survival curves: (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous media saturated with N2O containing 4×10–2 mol•l–1 ethanol (pH≅7.4) in the presence of: buffer (A), 1×10–5 mol•l–1 E1 (B), 1×10–5 mol•l–1 vitamin C (C) and mix of 1×10–5 mol•l–1 E1 and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

with the behavior compared to the action of TES transients as well as of those resulting from TES + vitamin C (see insert, Figure 1) in media saturated with air.

In the E1/vitamin C system (Figure 5, curve D) the main active primary agents are OH and the peroxide species $O_2^{\bullet-}$ and $^{\bullet}O_2C_2H_4OH$, which lead to negative ΔD_{37} values, demonstrating a strong cytostatic property. This is similar to the results obtained in the TES experiments performed under the same conditions.

The observed survival curves of *E. coli* bacteria using E1/vitamin C system in media saturated with N_2O, result from the action of OH, H and $^{\bullet}C_2H_4OH$ species (see Eqtn 2-4). The calculated ΔD_{37}(Gy) values of all curves exhibit a stronger proliferative effect (Insert, Figure 6), similarly to the corresponding data of TES (see Figure 2), which underlines the strong oxidizing effect of OH radicals.

Even a stronger proliferative effect of the same system was observed in media saturated with argon, where the reacting radicals are H, OH, e_{aq}^- and $^{\bullet}C_2H_4OH$ transients (insert, Figure 7).

Summing-up it can be stated that the obtained results from the E1/vitamin C system, using *Escherichia coli* as a biological model, demonstrate that the mentioned radicals lead practically to the same effect, as the one observed for TES under the corresponding conditions.

E1/vitamin C system: HPLC analysis/E1 regeneration. The degradation process of the E1 and vitamin C system, individually as well as a mixture, was likewise studied as a function of the absorbed radiation dose. The course for the corresponding curves were similar to those mentioned for the TES/vitamin C system (Figure 4). For the E1/vitamin C mixture, a regeneration of E1 transients by electron transfer from vitamin C acting as electron donor, was detected by using HPLC method.

The observed E1 regeneration process (82.6%) and the vitamin C consumption (18.7%) are in fair agreement with previously reported data, namely E1 regeneration (90.9%) and vitamin C consumption (15.4%) (17).

Discussion

In the *in vitro* studies (*E. coli* AB 1157) using TES in aerated aqueous media containing alcohol, the primary radicals OH, H and e_{aq}– (Eqtn 1) were partly converted into the acting $O_2^{\bullet-}$, $^{\bullet}C_2H_4OH$ and $^{\bullet}O_2C_2H_4OH$ transients. Under these conditions

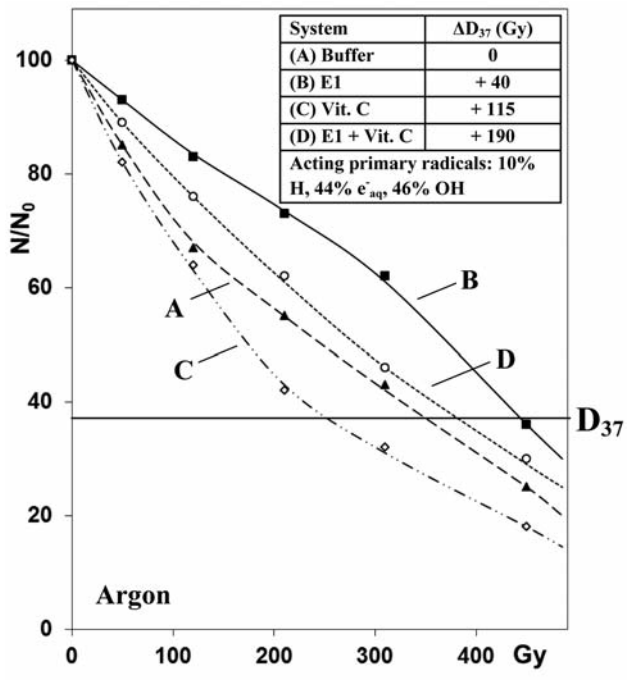

Figure 7. *Survival curves: (N)(N0) ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli (AB 1157) in aqueous media saturated with argon, containing 4×10^{-2} mol•l^{-1} ethanol (pH≅7.4) in the presence of: buffer (A), 1×10^{-5} mol•l^{-1} E1 (B), 1×10^{-5} mol•l^{-1} vitamin C (C) and a mix of 5×10^{-5} mol•l^{-1} E1 and vitamin C (D). Insert: ΔD37(Gy) values, calculated from the corresponding survival curve.*

the observed ΔD_{37}(Gy) values of TES indicated cytostatic properties. These values are even greater in the presence of vitamin C, used as an electron donor. In media saturated with N_2O, however, the acting transients are mainly $^{\bullet}C_2H_4OH$ radicals, in addition partly of OH and H, which induce the formation of hormone transients with radiation-protection properties. This effect is further increased in airfree medium, where the species e_{aq}^- and $^{\bullet}C_2H_4OH$ and in part OH and H react with TES. As a conclusion, it can be stated that oxidizing attacking radicals (*e.g.* $O_2^{\bullet-}$, $^{\bullet}O_2C_2H_4OH$) initiate the formation of TES intermediates with cytostatic properties, whereas the acting reducing species (e_{aq}^-, H, $^{\bullet}C_2H_4OH$) result in production of radiation-protecting hormone transients.

The investigations *in vitro* with E1 under the same conditions led to similar results, although the molecular structure of E1 is quite different from that of TES. The only exception is the ΔD_{37}(Gy) value obtained for E1 when *E. coli* were irradiated alone in aerated media (Figure 5, insert). In both studied systems: TES/vitamin C and E1/vitamin C the observed consumption of vitamin C was not adequate for corresponding hormone regeneration. This can be explained by the fact that the main vitamin C degradation product is dehydroascorbate, which is an even stronger electron donor than vitamin C (27).

Conclusion

Summing-up the *in vitro* results for both hormones, TES and E1, it can be stated that independent of the hormones' molecular structure, the determining factor of the biological properties of the resulting hormone intermediates is the chemical nature (oxidizing/reducing) of the radicals reacting with the hormones.

The specific UV-induced degradation of TES and vitamin C studied individually leads to regeneration of TES when both are irradiated in mixture as consequence of electron transfer from vitamin C to TES transients. TES regeneration was only found when TES transients were still in *statu nascendi*. Both processes, degradation (formation of transients) and regeneration (by electron transfer from vitamin C) are in state of a kinetic competition. Similar degradation/regeneration effects were also observed in experiments with E1 and vitamin C. It is interesting that in both studies of TES/vitamin C and E1/vitamin C systems, vitamin C acts as an electron donor but its consumption is not adequate for hormone regeneration. This, however, may be explained by the fact that in addition to vitamin C, dihydroascorbate (main product of the ascorbate radicals, AH•) acts as a very efficient electron donor (27). The exact reaction mechanisms in this case are not entirely clear.

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