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Mutual Interaction of 17β-Estradiol and Progesterone: Electron Emission. Free Radical Effect Studied by Experiments *In Vitro*

NIKOLA GETOFF[1], HEIKE SCHITTL[1], JOHANNES HARTMANN[2], MARION GERSCHPACHER[2], SHAOBIN YING[1], IREN DANIELOVA[2] and JOHANNES C. HUBER[2]

[1]*Section of Radiation Biology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, Austria;*
[2]*Department of Gynecologic Endocrinology and Reproduction, Medical University of Vienna, A-1090 Vienna, Austria*

Abstract. *Background: Based on the different behaviour of 17β-estradiol ($17\beta E_2$) and progesterone (PRG), it was of interest to investigate the interaction of both hormones in respect of their electron emission and cytotoxicity by experiments in vitro. Materials and Methods: The studies include determination of emitted electrons (e^-_{aq}) by the individual hormones as well as by their mixtures, all complexed with cyclodextrin (HBC). Experiments in vitro (Escherichia coli bacteria) were performed for a better understanding of the mechanisms involved. Survival ratios, ΔD_{37}(Gy), were calculated. Results: Aqueous HBC as well as $17\beta E_2$ and PRG, individually as well as in mixtures, are able to emit e^-_{aq}. The resulting transients can lead to the formation of metabolites, some of which can initiate cancer. It was established that both hormones, $17\beta E_2$ and PRG, interact in respect to their electron emission property. In the frame of experiments in vitro, it was found that oxidizing radicals (OH, $O_2^{\bullet-}$) lead to negative ΔD_{37}(Gy) values, indicating cytostatic properties. On the other hand, the primary reducing radicals (e^-_{aq}, H) lead to positive ΔD_{37}(Gy) values, indicating a radical-scavenging effect. Conclusion: The main outcome of this work is that PRG in combination with $17\beta E_2$ can strongly reduce the number of carcinogenic $17\beta E_2$-metabolites. This fact offers a new pathway for application of hormones in medical treatment of patients.*

It was recently proven for the first time that sexual hormones, such as 17β-estradiol ($17\beta E_2$) and progesterone (PRG) (1) as well as testosterone (TES) (2), can eject electrons (e^-_{aq}) in polar media containing water. This fact demonstrates the capability of these hormones to communicate with other biological systems in an organism by electron transfer processes *via* the brain-receiving centres without forming complexes with receptors (3). The same property was subsequently also observed for other hormones, *e.g.* the phytohormone genistein (4), 4-hydroxyestrone (5) and adrenaline (6). Thereby, a small fraction of the ejected e^-_{aq} is consumed by reaction with the hormones themselves (7). The hormone transients resulting from the electron emission process subsequently form various metabolites, some of which can initiate cancer (8-11). Thereby, those metabolites originating from PRG show fewer carcinogenic properties in comparison to those of $17\beta E_2$ (1, 10, 11). On the other hand it should be mentioned that PRG is also able to influence the biological properties of other hormones (12-14). Preliminary experiments *in vitro* using mixtures of PRG with $17\beta E_2$, with estriol or with estrone showed that PRG can very strongly influence the carcinogenity of hormone metabolites. These observations offer new pathways for a better understanding of certain biological processes resulting from the interaction of hormones.

As is well known, oxidizing (OH, $O_2^{\bullet-}$, *etc.*) as well as reducing free radicals (e^-_{aq}, H, $R^{\bullet}$, *etc.*) are permanently generated and consumed in living organisms. They can initiate a variety of biological processes (15). The action of free radicals on water-soluble $17\beta E_2$ (embedded in 2-hydroxypropyl-β-cyclodextrin; HBC) and on HBC was studied by experiments *in vitro* (model: *Escherichia coli* bacteria, AB1157) (16). HBC is a polysaccharide existing in various forms and plays an important role in nutrition. In aerated media (46% OH, 54% $O_2^{\bullet-}$), both substrates ($17\beta E_2$/HBC and HBC) act as efficient radical scavengers, whereby the effect of HBC is three times higher than that of $17\beta E_2$/HBC. However, the action of 90% OH and 10% H is even stronger in this respect. In air-free, aqueous media (44% $O_2^{\bullet-}$, 10% H, 46% OH), HBC, as well as

Correspondence to: Professor N. Getoff, Section of Radiation Biology, Faculty of Life Sciences, University of Vienna, Althanstr. 14, UZA II, A-1090 Vienna, Austria. Tel: +43 1427754966, Fax: +43 1427754965, e-mail: nikola.getoff@univie.ac.at

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17βE$_2$/HBC, exhibits a strong cytostatic effect. The (ΔD_{37})value of HBC, representing the difference of (ΔD_{37})HBC minus (ΔD_{37})buffer, is about double the value of that of 17βE$_2$/HBC. The reaction mechanism, however, is rather complicated, but it can be stated that the A-ring of the 17βE$_2$ molecule is the determining factor in the process: (i) as an electron donor and (ii) by leading to metabolites resulting from the various mesomeric phenoxyl-type structures, some of which can initiate cancer (1, 4, 15). It should also be mentioned that the degradation of 17βE$_2$, using UV light of a wavelength of 254 nm, results in a low quantum yield, Q=0.067 (17), which is very close to that previously determined for phenol (18).

Based on these data and experiences, the present study was focused on (i) the effect of HBC in combination with 17βE$_2$ and PRG in respect to cytotoxicity; (ii) the interaction of PRG with 17βE$_2$, both embedded in HBC, which makes them water soluble; (iii) the electron emission of these systems when excited in the singlet-state; as well as (iv) the effect of oxidizing (OH, $O_2^{\bullet -}$) and reducing free radicals (e^-_{aq}, H) on these systems studied by experiments *in vitro*.

Materials and Methods

Chemicals of highest purity available (≥99%; Sigma-Aldrich, Vienna, Austria) were applied as received. 17βE$_2$ and PRG were used as a water-soluble HBC complex. Triple-distilled water was used as solvent for preparation of the various media. For excitation of the substrates to the singlet state, a low-pressure Hg-UV lamp (HNS 12, OSRAM, 12 W) with incorporated VYCOR-filter for removal of the 185 nm line was used (19). The lamp was mounted in a special 4л-geometry irradiation double-wall vessel and connected to a thermostat to maintain the desired temperature of the solution during the experiment. Under these conditions the lamp provided monochromatic UV light (λ=254 nm; 4.85 eV/hν) with an intensity of 1×10^{18} hν/ml/min at 37°C. The emitted solvated electrons (e^-_{aq}) from the substrates were specifically scavenged by 1×10^{-2} mol/l chloroethanol (20) according to the following equation.

$ClC_2H_4OH + e^-_{aq} \rightarrow Cl^- + {}^{\bullet}C_2H_4OH$
($k=6.9\times10^9$ l/mol/s) (Eqtn 1)

Hence: $Q(Cl^-)=Q(e^-_{aq})$ (Eqtn 2)

The effect of the oxidizing and reducing free radicals on HBC, 17βE$_2$/HBC, PRG/HBC and 17βE$_2$/PRG/HBC mixtures were determined by experiments *in vitro*. As a model for living systems *E. coli* bacteria (AB1157) were used. The handling of the bacteria and the evaluation of the obtained results was previously described (16). The free radicals were generated by treating the aqueous systems with γ-ray under appropriate conditions at room temperature. A Gammacell 220 (Nordion Corp., Canada) instrument served as radiation source. Its dose rate (Gy/min) was determined and periodically controlled by modified Fricke dosimeter (21).

Results and Discussion

Electron emission. Certain organic compounds in aqueous media can emit electrons (e^-_{aq}) by excitation at the corresponding singlet state in competition to fluorescence (22). In the present case, the applied monochromatic UV light (λ=254 nm) fulfils this requirement. Using 1×10^{-4} mol/l HBC, the photo-induced emission of e^-_{aq} is presented in Figure 1 as a function of the absorbed UV dose.

The wave-like course of the curve illustrates the involvement of multi stage processes following the excitation of HBC molecules. This signifies that by electron emission HBC radicals are formed, which leads to products having the ability to eject electrons. Under the given experimental conditions, these can be determined after achieving a certain concentration. However, the yield of e^-_{aq} decreases slowly because of the reduced substrate concentration as well as of the photolytic products. This fact is also demonstrated by the obtained quantum yields, $Q(e^-_{aq})$, at the individual maxima, given as inset (I) in Figure 1. The electron emission process is escorted by a pH decrease with rising UV dose (inset II, Figure 1), which hints that the electron ejection results predominantly from the OH groups of the HBC molecule:

e.g. $ROH \rightarrow (ROH)^* \rightarrow RO^{\bullet} + H+ + e^-_{aq}$ (Eqtn 3)

The produced $RO^{\bullet}$ dextrin radicals can take part in subsequent processes.

Figure 2A shows the course of the electron emission of 1×10^{-4} mol/l 17βE$_2$ complexed with 3.98×10^{-4} mol/l HBC under the same experimental conditions as before. The curve passes through two maxima in the studied UV dose range, whereby the $Q(e^-_{aq})$ value decreases with increasing absorbed UV dose (Figure 2A, inset). However, using 1×10^{-4} mol/l PRG complexed with 2.72×10^{-4} mol/l HBC, depicted in Figure 2B, the curve shows three maxima, however, over a much larger UV dose. The $Q(e^-_{aq})$ values are more than ten times lower (Figure B, inset). This effect has been previously observed (1, 2) and is explained by the formation of unstable hormone complexes (associates), which consume a part of the emitted e^-_{aq}.

Concerning the $Q(e^-_{aq})=7.6\times10^{-2}$ of 17βE$_2$ and $Q(e^-_{aq})=2.8\times10^{-4}$ of PRG, previously determined in water-ethanol mixture using 1×10^{-5} mol/l hormone, the large difference of e^-_{aq} yields was attributed to the specific molecular structure of both hormones (1). Since the electron emission occurs predominantly from the A-ring of the 17βE$_2$ molecules, a phenoxyl radical type is formed (existing in several mesomeric forms), whereas PRG results in a radical cation ($PRG^{\bullet +}$). This postulation is supported by the previously reported quantum yield of 17βE$_2$ photo-degradation using UV light of 254 nm, $Q(17\beta E_2)=6.7\times10^{-2}$ (17), as well as $Q(17\beta E_2)=4.3\times10^{-2}$ (23). The quantum yields

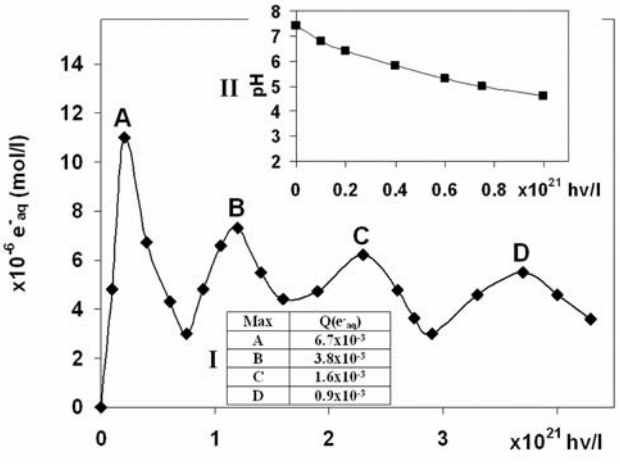

Figure 1. *Emission of electrons (e^-_{aq}) by UV irradiation (λ=254 nm) of 1×10^{-4} mol/l HBC in air-free, aqueous solution (pH ~7.4). The quantum yields of the ejected electrons, $Q(e^-_{aq})$, at the maxima are given as inset I. The pH as a function of the absorbed UV dose is shown in inset II.*

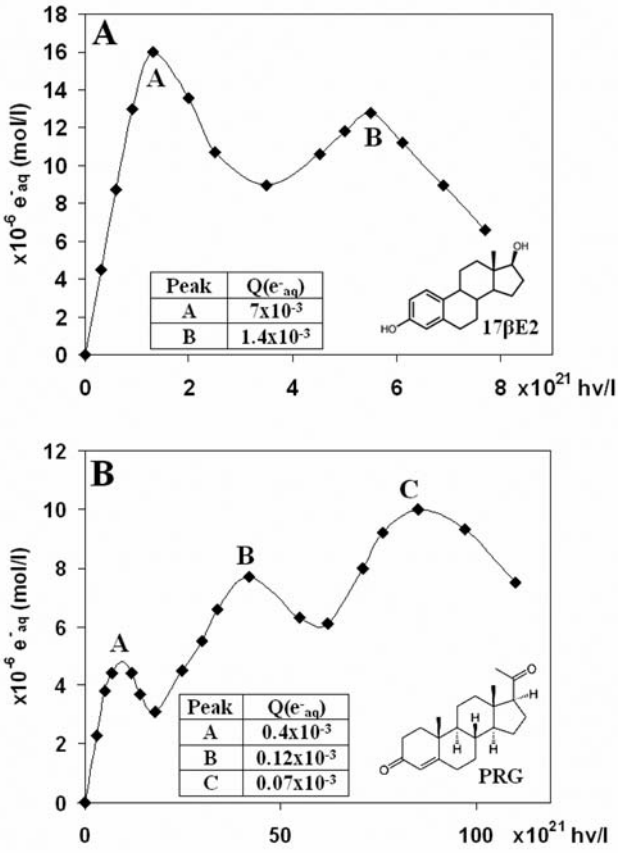

Figure 2. *Electron emission (e^-_{aq}) by irradiation of air-free, aqueous solution (pH ~7.4) with monochromatic UV light (λ=254 nm) of: A: 1×10^{-4} mol/l $17\beta E_2$ with 3.98×10^{-4} mol/l HBC and B: 1×10^{-4} mol/l PRG with 2.72×10^{-4} mol/l HBC as a function of the absorbed dose (hv/l). The calculated quantum yields, $Q(e^-_{aq})$, are given as insets.*

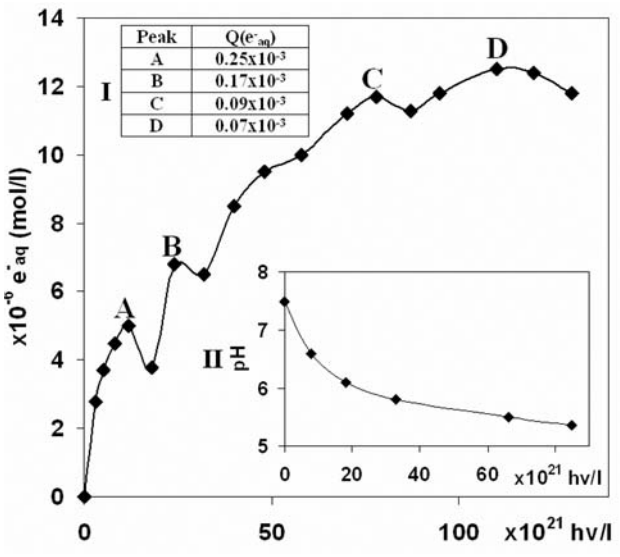

Figure 3. *Emission of electrons (e^-_{aq}) by irradiation of air-free, aqueous solution (pH ~7.4) of 1×10^{-4} mol/l $17\beta E_2$ + 1×10^{-4} mol/l PRG + 6.7×10^{-4} mol/l HBC with monochromatic UV light (λ=254 nm) as a function of the absorbed dose (hv/l). Inset I: $Q(e^-_{aq})$ values at the peaks; II: pH as a function of UV dose.*

are very close to these previously determined for phenol (18). It should be mentioned that with increasing absorbed UV dose, the pH of the media decreases in both systems, indicating that the electron emission occurs by involvement of the OH group of the A-ring of the molecule (1).

The influence of PRG on $17\beta E_2$ in the presence of HBC in respect to electron emission was studied by using 1×10^{-4} mol/l $17\beta E_2$ and 1×10^{-4} mol/l PRG with corresponding 6.7×10^{-4} mol/l HBC. The observed e^-_{aq} yield as a function of absorbed UV-quanta is presented in Figure 3. The curve shows a permanent increase with several small maxima (A-D). Clearly the determined Q-yields, representing overall data, result from the emitted and consumed e^-_{aq} from all three substrates. The calculated $Q(e^-_{aq})$ values at the maxima (Figure 3, inset I) are, however, lower, compared to those observed with the individual systems (Figure 1 and 2). In the present case, several simultaneously proceeding reactions are involved: (i) ejection of e^-_{aq} of each system, and (ii) partly consumation of e^-_{aq} by the substrates, since: $k(17\beta E_2+e^-_{aq})=2.7\times10^{10}$ l/mol/s (24), $k(PRG+e^-_{aq})\sim4\times10^9$ l/mol/s (2) and $k(HBC+e^-_{aq})=8\times10^7$ l/mol/s (25) in competition with scavenging of e^-_{aq} by chloroethanol, $k(ClC_2H_4OH+e^-_{aq})=6.9\times10^9$ l/mol/s (25). The products resulting from all these processes can naturally also participate to some extent in the processes, depending on their reaction rate constants and concentration. It is obvious that the biological efficiency of both hormones, $17\beta E_2$ and PRG, and their mutual influence, *e.g.* on the formation of

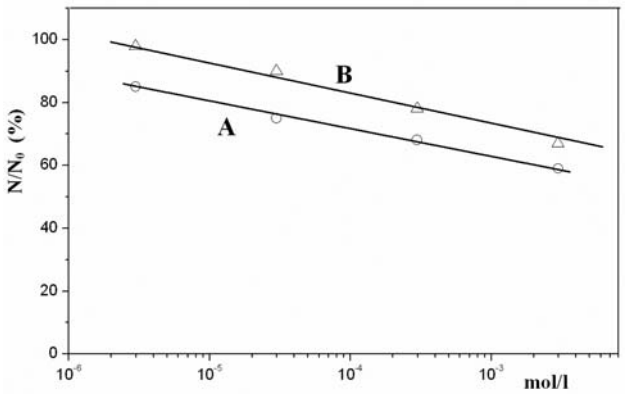

Figure 4. *Toxicity (%) to Escherichia coli bacteria (AB 1157) in aerated, aqueous media (pH~7.4) as a function of substrate concentration (mol/l) of HBC (A) and HBC and PRG (B).*

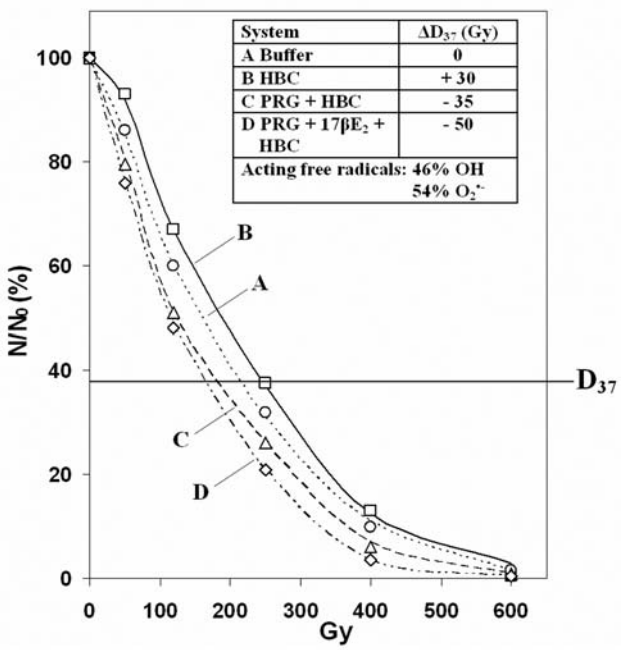

Figure 5. *Survival curves: N/N_0 ratio as a function of absorbed γ-radiation dose (Gy) of Escherichia coli bacteria (AB1157) in aqueous aerated media (pH~7.4) in the presence of: A: buffer; B: 2.72×10^{-4} mol/l HBC; C: 1×10^{-4} mol/l PRG + 2.72×10^{-4} mol/l HBC; and D: 1×10^{-4} mol/l PRG + 1×10^{-4} mol/l $17\beta E_2$ + 6.7×10^{-4} mol/l HBC. Inset: ΔD_{37} values (Gy), calculated from the corresponding survival curves.*

cancer-initiating metabolites, depends on the availability of certain compounds in the media. Evidently, the involved mechanisms are very complicated. Nevertheless, the specific molecular structure of PRG resulting in radical cation ($PRG^{\bullet+}$) formation after electron emission becomes apparent (1). It might be mentioned finally, that with ongoing electron emission, the pH of the aqueous medium decreases (*cf.* Figure 3, inset II). This indicates that the OH groups of HBC (*cf.* equation 3), as well as of the phenolic ring A of $17\beta E_2$ (equation 4) and of $PRG^{\bullet+}$ (equation 5), are the corresponding sources for H^+ formation:

$$17\beta E_2 \rightarrow 17\beta E_2^* \rightarrow 17\beta E_2^{\bullet} + e^-_{aq} + H^+ \quad \text{(Eqtn 4)}$$

$$PRG \rightarrow PRG^* \rightarrow e^-_{aq} + PRG^{\bullet+} + H_2O \rightarrow H^+ + PRG\text{-}OH \quad \text{(Eqtn 5)}$$

Experiments in vitro. Since organisms permanently generate oxidizing and reducing free radicals, which play an essential and many-sided role in various processes, it was of interest to investigate the mutual effect of $17\beta E_2$ and PRG encapsulated in HBC in the frame of experiments *in vitro*. *E. coli* bacteria (AB1157) served as a model in aqueous media (pH~7.4).

The primary free radicals resulting by γ-radiolysis of water, their yields and important conversion reactions are presented as follows (15):

$$H_2O \rightarrow e^-_{aq},\ H^{\bullet},\ OH^{\bullet},\ H_2,\ H_2O_2,\ H^+_{aq},\ OH^-_{aq} \quad \text{(Eqtn 6)}$$
G-value (2.7) (0.6) (2.8) (0.45) (0.72) (3.2) (0.5)

These species are active in air-free, aqueous solutions.
In the presence of air, H and e^-_{aq} are converted into peroxyl radicals:

$$H + O_2 \rightarrow HO_2^{\bullet} \quad (k=2\times10^{10}\ l/mol/s) \quad \text{(Eqtn 7)}$$
$$e^-_{aq} + O_2 \rightarrow O_2^{\bullet-} \quad (k=1.9\times10^{10}\ l/mol/s) \quad \text{(Eqtn 8)}$$
$$HO_2^{\bullet} \leftrightarrow H^+ + O_2^{\bullet-} \quad (pK=4.8) \quad \text{(Eqtn 9)}$$

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

$$e^-_{aq} + N_2O \rightarrow OH + OH^- + N_2 \quad (k=0.9\times10^{10}\ l/mol/s) \quad \text{(Eqtn 10)}$$

The toxicity (%) to the bacteria in aqueous, aerated media (pH~7.4) was studied as a function of substrate concentration from 5×10^{-6} up to 5×10^{-3} mol/l HBC (Figure 4). The HBC concentration used for the survival curves had a toxicity of less than 30%, whereas for PRG/HBC, the toxicity was about 20%. For 1×10^{-4} mol/l $17\beta E_2$/HBC there was no toxicity observed at all (not given in Figure 4).

In aerated media, the survival curves of the bacteria (N/N_0 ratio) are presented as a function of the absorbed radiation dose (Gy) for each individual system (Figure 5). As expected, increasing radiation dose led to a decrease of N/N_0 values. In the present case, the reducing radicals H and e^-_{aq} are converted into peroxyl radicals within a few μs (*cf.* equation 7-9). Therefore, the acting free radicals are 46% OH and 54% $O_2^{\bullet-}$. The ΔD_{37} values calculated represent the

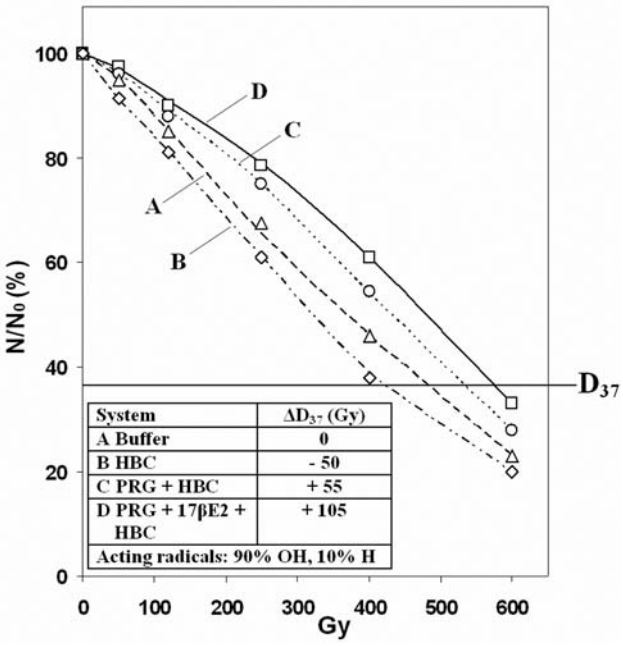

Figure 6. *Survival curves: N/N_0 ratio as a function of absorbed γ-ray dose (Gy) of E. coli bacteria (AB1157) in aqueous solution saturated with N_2O (pH~7.4) in the presence of: A: buffer; B: 2.72×10^{-4} mol/l HBC; C: 1×10^{-4} mol/l PRG + 2.72×10^{-4} mol/l HBC; and D: 1×10^{-4} mol/l PRG + 1×10^{-4} mol/l $17\beta E_2$ + 6.7×10^{-4} mol/l HBC. Inset: ΔD_{37} values (Gy), calculated from the corresponding survival curves.*

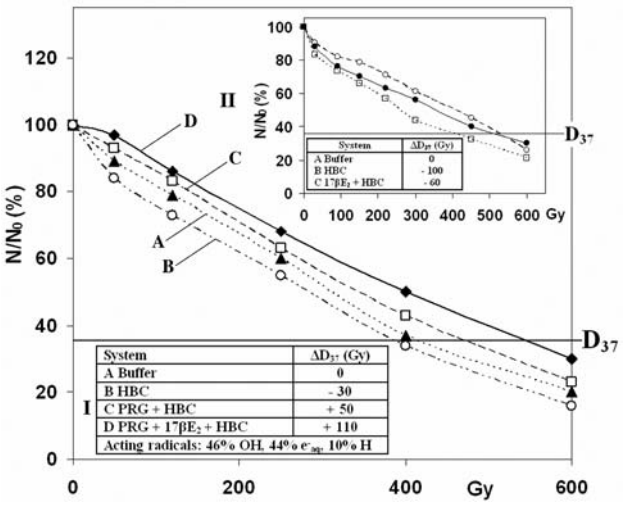

Figure 7. *Survival curves: N/N_0 ratio as a function of absorbed γ-ray radiation dose (Gy) of Escherichia coli bacteria (AB1157) in aqueous media (pH~7.4), saturated with argon in the presence of: A: buffer; B: 2.72×10^{-4} mol/l HBC; C 1×10^{-4} mol/l PRG + 2.72×10^{-4} mol/l HBC; and D: 1×10^{-4} mol/l PRG + 1×10^{-4} mol/l $17\beta E_2$ + 6.7×10^{-4} mol/l HBC. Inset I: ΔD_{37} values (Gy) of the given systems; II: Survival curves: N/N_0 ratio as a function of absorbed radiation dose (Gy) of the same bacterial type in media saturated with argon in the presence of: A: buffer; B: 3.98×10^{-4} mol/l HBC; C: 1×10^{-4} mol/l $17\beta E_2$ + 3.98×10^{-4} mol/l HBC and calculated ΔD_{37} values of the survival curves.*

radiation dose at which N/N_0=0.37 and were taken from the corresponding curves. The ΔD_{37}(Gy) data were obtained by subtracting D_{37} buffer from each individual D_{37} value: D_{37}(Gy) sample-D37(Gy) buffer=ΔD_{37}(Gy) of sample. Positive ΔD_{37}(Gy) values indicate a radiation (free radicals) protective property of the system, whereas negative values demonstrate cytostatic ability of the substrate. The calculated ΔD_{37}(Gy) values of all curves are given as an inset in Figure 5. The positive ΔD_{37}(Gy) value of curve B indicates that HBC is a good scavenger for oxidizing radicals. Hence, HBC acts as a protector against OH and peroxyl radicals. The ΔD_{37}(Gy) values of curves C and D are negative, demonstrating their cytostatic effect, whereby the system PRG plus $17\beta E_2$ plus HBC has much stronger capability in this respect than the mixture of PRG and HBC.

In Figure 6, the survival curves obtained for the same systems, but in media saturated with N_2O, are presented. In this case, the e^-_{aq} is converted into OH radical. Since the reaction rate constants of e^-_{aq} with both hormones are very high, the reaction of e^-_{aq} with hormones cannot be completely neglected. Under these conditions, the ΔD_{37}(Gy) values show an opposite effect compared to the data obtained in aerated media. Namely, ΔD_{37}(Gy) of HBC is negative, whereas the corresponding values of the systems shown in Figure 6C and D are positive. This surprising effect can be partly attributed to the competitive action of H atoms as well as to the action of hormone intermediates and to the resulting products. Involvement of e^-_{aq} may also contribute to the process. Likewise, the transients originating from HBC, the concentration of which is higher than that of the hormones, certainly contribute to the observed effect.

The above postulation seems to be confirmed from the data obtained by the survival curves of the bacteria treated with γ-ray in media saturated with argon, where the acting free radicals are 10% H, 44% e^-_{aq} and 46% OH (Figure 7). The calculated ΔD_{37}(Gy) values (Figure 7, inset I) are very similar to those given in the inset of Figure 6. This hints at the determining role of the reducing species (e^-_{aq}, H, *etc.*) in the course of the biological processes. On the other hand, it is interesting that under the same experimental conditions, the system of $17\beta E_2$ with HBC had a strong cytostatic property (Figure 7, inset II) in contrast to the system of PRG with HBC. These results underline once more the contrary biological action of $17\beta E_2$ compared to PRG under the given conditions. On the other hand, it seems that HBC as a representative of the various types of polysaccharides which exist, can influence, at least to some extent, the biological action of the hormones and their metabolites, depending on the environment.

Conclusion

The subject matter of the present work embraces some biological consequences of the mutual interaction of $17\beta E_2$ and PRG in the presence of HBC in aqueous media. Three main aspects are highlighted: (i) The role of HBC as a food representative and its influence in the electron emission process of $17\beta E_2$ and PRG, as well as of their mixtures; (ii) investigations into the effect of reducing and oxidizing free radicals on the aforementioned systems by experiments *in vitro* with respect to possible cancer initiation by hormone metabolites; and (iii) the influence of HBC on the biological action of hormones and their metabolites. The results are summarized as follows:

Aqueous HBC leads to formation of solvated electrons (e^-_{aq}) by excitation. At the same time the resulting products of HBC are likewise able to emit e^-_{aq}.

The $Q(e^-_{aq})=7x10^{-3}$ determined for 1×10^{-4} mol/l $17\beta E_2$ with 3.98×10^{-4} mol/l HBC is practically the same as previously found for $17\beta E_2$ alone in a water-ethanol mixture under otherwise identical conditions (1). This effect can be explained by the fact that part of the emitted e^-_{aq} is simultaneously consumed by the substrate mixture.

The same effect, emitting and consuming e^-_{aq}, is also observed for the PRG with HBC system.

The mixture of all three substrates delivers even a smaller e^-_{aq} yield compared to the previously reported individual values for both hormones (1). This fact demonstrates the strong influence of PRG in the mixture with $17\beta E_2$. As a consequence of this, fewer carcinogenic metabolites are expected to be formed by application of a mixture of both hormones.

The effect of free radical action (e^-_{aq}, H, OH, $O_2^{\bullet-}$, *etc.*) on the individual as well as on mixed systems is rather complicated since several factors are responsible for the observed effect, such as individual substrate concentration and reactivity towards the single radicals, mutual involvement of the starting substrate as well as of the resulting degradation hormone products *etc.*

In the presence of air (46% OH, 54% $O_2^{\bullet-}$), the calculated ΔD_{37}(Gy) values on the basis of the survival curves show that HBC acts as a successful scavenger of the radicals (radiation protector). On the other hand, the PRG with HBC system, as well as that of PRG with $17\beta E_2$ and HBC, exhibits negative ΔD_{37}(Gy) values, which indicates a cytostatic action. Here again, the influence of PRG as a supplier of fewer cancer-initiating metabolites predominates.

In media saturated with N_2O (acting radicals: 90% OH, 10% H), the ΔD_{37}(Gy) values are completely opposite, probably due to the reducing properties of H atoms and involvement of the resulting radiolytic products.

In air-free media (10% H, 44% e^-_{aq}, 46% OH), the D_{37}(Gy) values are practically the same, supporting the notion of the strong involvement of the reducing species (H and e^-_{aq}) in the formation of carcinogenic metabolites.

The obtained results concerning the mutual influence of $17\beta E_2$ and PRG, complexed with HBC, in respect to metabolite formation as a consequence of electron emission (e^-_{aq}) as well as by attack of oxidizing and reducing free radicals are rather complicated. However, the main outcome from the obtained results is that the presence of PRG in a mixture with $17\beta E_2$ can reduce the numerous carcinogenic metabolites originating from $17\beta E_2$ and that HBC can influence the biological action of hormones, depending on the environment in which it acts.

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