Abstract. Cytochrome c (Cytc) in airfree, aqueous media (pH ~7.4; 37˚C) can emit e\textsuperscript{−aq} when exited in single state by monochromatic UV light (λ=254 nm). The obtained Qi(e\textsuperscript{−aq}) at lower Cytc concentrations is higher compared to the amount obtained at higher substrate concentrations because at >10\textsuperscript{−8} mol l\textsuperscript{−1} Cytc ‘associates’ (unstable complexes) are formed, which consume a part of the ejected e\textsuperscript{−aq}. The primary, as well as secondary, photolytic products of the substrate likewise emit e\textsuperscript{−aq}, but with much lower Q(e\textsuperscript{−aq}) values. On the other hand, the photolysis Q(Cytc) values are lower than those of Q(e\textsuperscript{−aq}), since Cytc transients can be regenerated by partial consumption of emitted e\textsuperscript{−aq}. In addition to this, the produced Cytc\textsuperscript{•+} (radical cations) can react with water, thus also regenerating Cytc.

By UV treatment of Cytc/vitamin C (VitC) mixtures, it was found that a mutual electron transfer process takes place. This process is in concordance with the corresponding reaction rate constants (k) of the substrates with e\textsuperscript{−aq}. In aerated aqueous solutions, the ejected e\textsuperscript{−aq} are converted into oxidizing O\textsubscript{2}\textsuperscript{•−} species, leading to substrate degradation. In addition, the Cytc transients resulting by e\textsuperscript{−aq} emission can be scavenged by oxygen and the obtained peroxyl radicals contribute to the degradation process. The Q(Cytc) values are similar to those obtained in airfree media. Both processes, regeneration and degradation of Cytc, are competing and are in dependence of the experimental parameters. The results are of interest for both biology and medicine.

Cytochrome c (Cytc) is a stable hemoprotein occurring in the cells of all aerobic organisms. It contains covalently bond heme \(c\) as a prosthetic group and is located at the outer surface of the inner mitochondrial membrane. Cytc plays a vital role in cellular oxidation and is regarded as an universal catalyst of the respiratory process (1-3). Because of its fluctuation within the cell between the ferrous and ferric states of the cytohemine, it can be classified as an efficient biological electron transporter (4). On the other hand, it is well known that aqueous ferro-ions can emit electrons (e\textsuperscript{−aq}, ‘solvated electrons’) by photo excitation, resulting in ferric ions observed in aqueous CO\textsubscript{2} reduction (5). Since Cytc acts as an efficient antioxidant (4) it was also interesting to study its ability to eject e\textsuperscript{−aq} in aqueous media by photo-excitation as well as the conditions determining the process. It has recently been reported that vitamin C (VitC) acts as a rather powerful electron donor in the regeneration of hormone transients in ‘statu noscendi’ state by the electron transfer process (6, 7). Therefore, the mutual electron interaction of Cytc and VitC was also investigated in order to compare their specific reduction power.

Materials and Methods

Triple-distilled water and chemicals of highest purity available (>99%; Sigma Aldrich, Vienna, Austria) were used for the preparation of the aqueous solutions. In order to remove oxygen, the solutions were saturated for 20 min with high purity argon (Messer A.G., Vienna, Austria) directly in the irradiation vessel. The specially designed 4π-geometry double-wall irradiation vessel was connected to a thermostat to maintain the desired temperature of the solution during the experiment (8). Monochromatic UV light of 254 nm (4.85 eV h\textnu–1) was provided by a low pressure Hg lamp (HNS 12, OSRAM, 12 W) with incorporated VYGOR filter for removal of the 185 nm line. The intensity of the lamp, \(I_0=1\times10^{-18} h\textnu ml\textsuperscript{−1} min\textsuperscript{−1}\), was determined by means of monochloric acetic acid actinometer (9). The emitted e\textsuperscript{−aq} of the substrate were scavenged by 1×10\textsuperscript{−2} mol l\textsuperscript{−1} chlorethanol (10), where:

\[
\text{ClC}_2\text{H}_4\text{OH} + e\textsuperscript{−aq} \rightarrow \text{Cl}\textsuperscript{−} + \text{•C}_2\text{H}_4\text{OH}
\]

\(k_1=6.4\times10^8 \text{ l mol}\textsuperscript{−1} s\textsuperscript{−1}\) (11) \(\text{Eq.1}\)

Hence, \(Q(\text{Cl}\textsuperscript{−})=Q(e\textsuperscript{−aq})\) \(\text{Eq.2}\)

Photo-induced degradation of separately UV-irradiated Cytc and VitC as well as their mixture, was followed by high performance liquid chromatography (HPLC). Samples were analyzed using a Hewlett-Packard Agilent 1100 HPLC series with a series 1050 diode
array detector commeted to a computer. The products were separated on an Agilent Poroshell 300 SB-C8 column (2.1×75 mm, 5 μm particle size) at a temperature of 30˚C. Samples of 20 μl were injected and elution was achieved by a linear gradient between mobile phase (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient started with 100% (A) decreasing to 0% in 5 min (100% B), held for 2 min with a flow rate of 1 ml per min. The detection of Cytc was performed at: 407, 409 and 414 nm and that of VitC at 250 nm.

Results and Discussion

Recently, it has been found that large organic molecules, such as hormones and related compounds in polar media, form ‘associates’ (unstable complexes) at concentrations >10⁻⁸ mol⁻¹ l⁻¹ (6, 7, 12). Hence, a similar study was performed using various concentrations of Cytc in aqueous solution (pH~7.4). In fact it was found, that the molar extinction coefficient at 254 nm (ε₂₅₄) of the solutions did not obey Lambert-Beer’s law. Consequently, Cytc also forms ‘associates’ at higher concentrations. The behavior of Cytc on this respect is expressed by the dependence of ε₂₅₄ values as a function of Cytc concentration as shown in Figure 1. This fact is rather important in correlation with the electron emission of Cytc.

The expectation that aqueous Cytc would eject electrons (e⁻ₐq) by excitation in singlet state (λ=254) was confirmed. Thereby it was found that the e⁻ₐq yield strongly depends on the applied Cytc concentration, where a higher Cytc concentration leads to lower e⁻ₐq yield because of ‘associate’ formation, which predominately comprises molecules in the ground state, partly consuming e⁻ₐq. This effect is demonstrated by e⁻ₐq Yield (mol l⁻¹) presented as a function of absorbed UV-quanta (hv l⁻¹) for two Cytc concentrations (Figure 2). Additionally, it was observed that primary, as well as secondary, photolytic products are also able to emit e⁻ₐq, but with essentially lower yields (see peaks 2 and 3 in Figure 2).

The e⁻ₐq emission from excited Cytc molecules can partly result from Fe²⁺ (5), as well as from the double bonds of heme groups and –COOH groups (13). However, due to the very high reaction rate constant (k) of Cytc with e⁻ₐq (see Table I), an essential proportion of the emitted e⁻ₐq is consumed by the molecules in the ground state as pointed out above (associates predominately comprise such molecules) hence the detected Qₐₑₐₐ(Qₑₐₑₐₐ) yield decreases with increasing Cytc concentration.

Figure 1. Molar extinction coefficient (ε₂₅₄; 1 mol⁻¹ cm⁻¹) in dependence of the Cytc concentration (mol l⁻¹) demonstrating the formation of Cytc associates in aqueous solution (pH~7.4).

Figure 2. Electron emission of airfree aqueous solution (pH~7.4) as a function of absorbed UVdose (hv l⁻¹). (A) 5×10⁻⁶ mol l⁻¹ Cytc; (B) 1×10⁻⁵ mol l⁻¹ Cytc. The calculated initial quantum yields, Q(e⁻ₐq)ₐ, at the corresponding peaks are given in the inset.
It is interesting to note, that the observed yield of Cytc photolysis, $Q_{\text{Cytc}}$, is much lower compared to $Q_{i(e^{-aq})}$ values determined for various substrate concentrations (Table II, Figure 3).

In order to explain this fact, the following considerations have been made: As already mentioned, Fe$^{2+}$ can emit $e^{-aq}$, thus oxidizing to Fe$^{3+}$ (5). Furthermore, the double bonds ($\pi$-electrons) of the heme systems are also possible sources for electron ejection, similar to progesterone and related hormones (6, 7). Thereby radical cation (Cytc$^{•+}$) is produced (eq.3). The Cytc$^{•+}$ species can subsequently react with water, regenerating Cytc, according to eq. 4.

\[
\text{Cytc} \xrightarrow{by} \text{Cytc}^{•+} \rightarrow e^{-aq} + \text{Cytc}^{•+} \quad \text{(Eq.3)}
\]

\[
\text{Cytc}^{•+} + H_2O \rightarrow \text{Cytc} + H^+ + OH^+ \quad \text{(Eq.4)}
\]

The irradiated solutions exhibited a pH decrease, depending on the absorbed UV dose. According to eq. 4, regeneration of Cytc can occur. Thereby the produced OH radicals are scavenged by chloroethanol:

\[
OH^+ + CIC_2H_4OH \rightarrow ClC_2H_2OH^+ + H_2O \quad (k_5 = 9.5 \times 10^8 \text{ mol}^{-1} \text{s}^{-1}) \quad \text{(11)}
\]

\[
OH^+ + \text{Cytc} \rightarrow \text{Cytc-OH (OH- adduct)} \quad \text{(Eq.6a)}
\]

\[
\rightarrow \text{Cytc}^{•} + H_2O \quad (k_6 = 1.4 \times 10^{10} \text{ mol}^{-1} \text{s}^{-1}) \quad \text{(11)}
\]
on the data of Table I, show the percentage of e\textsuperscript{−aq} scavenged in various mixtures of chloroethanol and CytC (Table III). Obviously, the e\textsuperscript{−aq} fraction reacting with CytC is much smaller compared to that of chloroethanol, but it increases with the concentration of CytC because of associate formation.

Based on all the above considerations, the rather low Qi(CytC) values in Table II are so far satisfactory explainable. CytC is also recognized as an efficient antioxidant (4), an ability which is now proven to be equivalent to its ability to emit e\textsuperscript{−aq}. Based on this fact, it was interesting to compare VitC and CytC with respect to the strength of their antioxidant behavior. For this purpose the photolysis of airfree aqueous solutions (pH~7.4; 37˚C) of 1×10\textsuperscript{−5} mol l\textsuperscript{−1} VitC and the same concentration of CytC were studied separately, as well as in a mixture of both. The individual substrate remainder (Rm, %) of each compound, applied solo or in mixture was analyzed by HPLC and was shown to be in dependence of the absorbed UV-quanta (hν l\textsuperscript{−1} at λ=254 nm) (Figure 4).

Curve A represents solo-photolysis of VitC and A1 indicates the remainder (%) of VitC in the mixture. The calculated Qi values are presented in the inset. Obviously, Qi(A1) is 49% smaller then Qi (A), indicating that VitC is strongly degraded in the mixture. This fact demonstrates that VitC transfers e\textsuperscript{−aq} to CytC transients, causing their regeneration. Curve B, representing solo-degradation of CytC, having Qi(Rm)=0.0295, and curve B1 showing the course of the individual CytC degradation in the mixture with VitC, results in Qi(Rm)=0.0270. This indicates that 8.5% of e\textsuperscript{−aq} emitted from CytC are transferred to VitC. These data clearly demonstrate that: (i) a mutual electron transfer takes place in the mixture of both substrates; (ii) the effect is in agreement with the reaction rate constants of e\textsuperscript{−aq} for the corresponding substrate (see Table I).

The photolytic products resulting from 1×10\textsuperscript{−5} mol l\textsuperscript{−1} CytC with 5×10\textsuperscript{−5} mol l\textsuperscript{−1} VitC in mixture, were also analyzed by HPLC. The course of the CytC degradation, as well as the formation of products (%), are presented as a function of the absorbed dose (hν l\textsuperscript{−1}) in Figure 5.

Curve A (Figure 5) represents the photolysis of CytC in the mixture, showing a slight increase at a dose of ~1.5×10\textsuperscript{21} hv l\textsuperscript{−1}, indicating complex formation with VitC. A specific curve of VitC photolysis could not be determined. At the same time, the formation of two products, P1 and P2, was registered, which were shown to be complexes of both CytC and VitC. This confirms the absence of the individual yield of VitC photolysis from the mixture. The yield of P1 complex passes a maximum at a UV dose of ~1.2×10\textsuperscript{21} hv l\textsuperscript{−1}, whereas the yield of P2 gradually increases with the absorbed dose. The calculated Qi values are presented in the inset in Figure 5.
This kind of experiments have been repeated using 1×10⁻⁵ mol l⁻¹ Cytc, but 1×10⁻⁴ mol⁻¹ l⁻¹ Vitc and a similar conclusion was derived. The calculated Q_i-values in this case are given as insert (II) in Figure 5.

Since in humans the oxidizing species (OH\(^•\), O\(_2\)^•⁻/HO\(_2\)^•, 
\(–\)ROO\(^•\), etc.) play an essential role, it was also interesting to study their effect on Cytc. The oxidizing radicals are produced as a consequence of the e\(^−\)aq emission in aerated, aqueous Cytc solutions (pH~7.4) namely:

\[
\begin{align*}
\text{O}_2 + \text{e}^{-\text{aq}} & \rightarrow \text{O}_2^{•−} \\
(\text{k}=2×10^{10} \text{ mol}^{-1} \text{ s}^{-1}) \ (11) \\
\text{O}_2 + \text{H} & \rightarrow \text{HO}_2^{•} \\
(\text{k}=1.9×10^{10} \text{ mol}^{-1} \text{ s}^{-1}) \ (11) \\
\text{HO}_2^{•} & \rightarrow \text{H}^{•} + \text{O}_2^{•−} \\
(\text{pK}=4.8) \ (14) \\
\end{align*}
\]

(Eq.7a) (Eq.7b) (Eq.8)

Based on the pK value of eq. 8 it is obvious that the O\(_2\)^•⁻ species are exclusively involved in the process.

Some results in this respect are presented in Figure 6 (part I and II) obtained by using 5×10⁻⁶ and 1×10⁻⁵ mol l⁻¹ Cytc in aerated aqueous solution (pH~7.4; 37°C). Figure 6 (I) shows the degradation course of Cytc for these concentrations and Figure 6 (II) the resulting product formation. The calculated Q_i values of the products expressed by the corresponding curves are given in the insets in Figure 6; it can be concluded that the products are mainly formed as a consequence of the reaction of O\(_2\)^•⁻ species produced by eq. 7a followed by reactions shown in eq. 9 and 10:

\[
\begin{align*}
\text{Cytc} + \text{O}_2^{•−} & \rightarrow \text{Cytc}^{•} + \text{HO}_2^{−} \quad \text{(Eq.9)} \\
\text{Cytc}^{•} + \text{O}_2 & \rightarrow \text{Cytc-O}_2^{•−} \rightarrow \text{Products} \quad \text{(Eq.10)} \\
\end{align*}
\]

(Adduct)

Naturally, in addition to O\(_2\)^•⁻ species other transients resulting from Cytc could, very likely, also be involved in the process, e.g.

\[
\text{Cytc} + \text{Cytc-O}_2^{•−} \rightarrow \text{Products} \quad \text{(Eq.11)}
\]

In other words, the reaction mechanism in the presence of air is rather complicated.

**Conclusion**

The highlights of Cytc investigations can be summarized into the following points: (i) at concentrations higher than 10⁻⁸ mol l⁻¹, Cytc forms ground state associates (unstable complexes), hence, solutions in this concentration range do not obey Lambert-Beer’s law. (ii) Cytc in aqueous, airfree solutions can emit e\(^−\)aq when excited in the singlet state (λ=254 nm; 4.85 eV hν⁻¹). (iii) With increasing Cytc concentration the Q_i(e\(^−\)aq) value decreases, because of the associates content, if the Cytc molecules in the ground state predominate, which consume a part of the ejected e\(^−\)aq. (iv) The Q_i yield of photolysis and product formation naturally depends on substrate concentration. (v) The primary and the secondary photolytic Cytc products also emit e\(^−\)aq but with much lower yields. (vi) In solutions containing both, Cytc and VitC, a mutual electron transfer process occurs, which is in agreement with the corresponding individual reaction rate constants (k) for the corresponding reactions with e\(^−\)aq. In this case, two kinds of products, P1 and P2, were formed showing different formation course.

Summing up, it should be stressed out that the various biological properties of Cytc obviously are based on its ability to eject, consume and transfer electrons to reaction partners and on the behavior of the resulting transients under different conditions.

\[
\begin{align*}
\text{Curve} & \quad \text{Cytc} \quad \text{mol l}^{-1} \quad \text{Q}_i \text{(Cytc)} \\
\text{A} & \quad 5 \times 10^{-6} \quad 0.0004 \\
\text{B} & \quad 1 \times 10^{-5} \quad 0.0013 \\
\text{Curve} & \quad \text{Cytc} \quad \text{mol l}^{-1} \quad \text{Q}_i \text{(Cytc)} \\
\text{A1} & \quad 5 \times 10^{-6} \quad 0.0005 \\
\text{B1} & \quad 1 \times 10^{-5} \quad 0.0018 \\
\end{align*}
\]
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References

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