Flash Photolysis and ESR Studies on Hematoporphyrin and Photofrin II in Polar and Microheterogeneous Environment

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Hematoporphyrin (HP) and its derivatives (HPD; e.g. photofrin II) are used as efficient sensitizers in the photodynamic treatment of tumors. The photoinduced formation of various transients resulting from these substrates at pH 12 and 7.4 were studied by conventional flash photolysis and by ESR techniques in the presence of various additives. EDTA is acting as an efficient reducing agent for the triplet state of the substrates. The absorption spectra of some transients ($HP^{\cdot -}$, HP–OH, $HPD^{\cdot -}$, HPD–OH) were obtained. Further, ESR studies (pH 4–7) showed that ^3HP is mostly converted into $HP^{\cdot -}$ in the presence of EDTA, but this is not the case with ^3HPD. In airfree HPD solutions a longlived transient was registered, which was attributed to $HPD^{\cdot +}$ species. In aerated substrate solutions the singlet oxygen ($^1O_2$) and peroxyradicals are formed. The discussed transients very likely are involved in the photodynamic treatment of tumors in man.

Key words: Hematoporphyrin (HP), Hematoporphyrin derivatives (HPD), Flash Photolysis, ESR, Free radicals.

Introduction

In recent years hematoporphyrin derivative (HPD) and especially its more purified version, identified as dihematoporphyrin ether [1] ester [2] (DHE) or their higher molecular mass oligomers (Fig. 1) have been examined as photodynamic agents for treatment of tumors in man. It is generally assumed that the tumor damage occurs via energy and/or less likely through charge transfer processes from porphyrin excited states to the biological material [3, 4]. It has been established that the desactivation of the first triplet state of hematoporphyrin ^3HP to the ground state occurs mainly by energy transfer to oxygen [5–7]. Thereby the quantum yield of singlet oxygen generation is found to be high ($Q = 0.4 - 0.6$ [6]) having an oxygen quenching constant $k(O_2) = 1 - 3 \times 10^9$ $M^{-1}\,s^{-1}$ [7]. On the other hand, the studies of the photophysical and photochemical properties of HP have shown that its photosensitizing activity depends on its aggregation state, polarity of the medium, presence of reducing agents etc. [8–10]. The assumption that the photodynamic effect is mainly due to the ^3HP initiated a number of flash photolysis and pulse radiolysis studies of hematoporphyrin and its related compounds [11–14]. The question, whether primary free radicals resulting from HP or related compounds are involved in the photosensitization reactions is, however, still open. In addition to this, data of the transients, obtained by flash photolysis, pulse radiolysis and ESR techniques, do not permit a direct comparison [11].

The aim of this work was to obtain additional spectroscopic and kinetic characteristics of the transients resulting from HP and DHE under influence of light. The effects of HP and DHE aggregation state, environment, presence of reducing agents, etc. were investigated applying common flash photolysis technique. In addition to this, ESR spectroscopy was used in order to get further data on porphyrin free radical formation. Special attention was given to the photoinduced generation of "solvated electrons" (e^-_{aq}) from HP and DHE, respectively, and the resulting radical cations, $HP^{+\cdot}$ and $DHE^{+\cdot}$.

Materials and Methods

Preparation of Solutions

The accepted structure formulas of the used substrates, HP and DHE are shown in Figure 1. In polar

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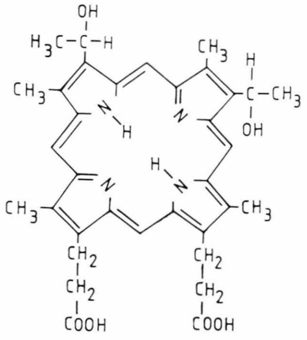
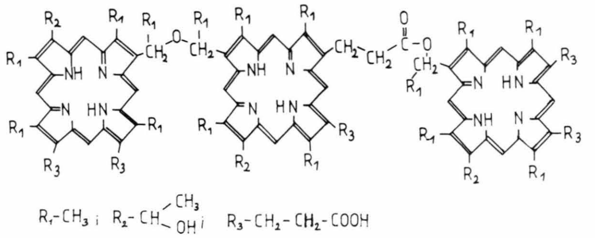


HEMATOPORPHYRIN (HP)



PHOTOFRIN II

Fig. 1. Structure formula of hematoporphyrin (HP) and photofrin II (HPD). (Hematoporphyrin ether/ester oligomer; HPD) [1].

environment, depending on concentration and protonation level HP is known to exist in different aggregate/monomer states. The covalently linked porphyrin molecules of DHE tend to aggregate even at conditions, where HP is monomeric. In the present work, using low substrate concentrations ($< 5 \times 10^{-6}$ M) in alkaline solutions (pH 12), or in the presence of Triton X 100 micelles (pH 7) the equilibrium

$$n\,\mathrm{HP} \rightleftharpoons (\mathrm{HP})_n \,. \qquad (1)$$

is preferentially shifted to the monomeric form.

Hematoporphyrin IX diHCl (MW 671.6; Koch Light Laboratories, England) and Hematoporphyrin derivative (DHE, averaged MW 1200; Photofrin II; Photomedia Inc., N.J., USA) were used as obtained. It might be mentioned that Photofrin II can contain up to 30% monomeric porphyrins, such as Hematoporphyrin (HP), Protoporphyrin (PP), Hydroxyethylvinyl-deuteroporphyrin (HVD), etc. For flash photolysis HP ($2-5 \times 10^{-6}$ M) and DHE ($1-2 \times 10^{-6}$ M) were dissolved in four times distilled water at pH 12 (adjusted with p.A. NaOH) or in 0.07 M phosphate buffer (pH 7.4). Further chemicals used were:

N_2O (2.8×10^{-2} M) for e^-_{aq} scavenging,

$$N_2O + e^-_{aq} \rightarrow OH + OH^- + N_2 \,, \qquad (2)$$

$$k_2 = 0.91 \times 10^{10}\ M^{-1}\,s^{-1} \quad [16],$$

t-butanol (10^{-1} to 10^{-2} M) as specific OH-scavenger

$$\text{t-}C_4H_9OH + OH \rightarrow \text{t-}C_4H_8OH + H_2O \,, \qquad (3)$$

$$k_3 = 5.5 \times 10^{8}\ M^{-1}\,s^{-1} \quad [17],$$

and high-purity argon for removing oxygen. In some experiments ethylenediaminetetraacetic acid (EDTA) (2×10^{-2} M) was applied as a reducing agent in order to obtain HP-radical anion ($HP^{\cdot -}$) [15].

Instrumentation

The applied flash photolysis equipment (Northern Precision [18]) has now been completely computerized (Digital Oscilloscope PM 3320/40 connected with Future AT-286), allowing sampling procedure. An air-filled flash lamp (20 mbar air) provided light pulses of 10 μs duration at 20–200 J discharge energy. The absorption spectra of the starting solutions were measured with a double-beam spectrophotometer (Hitachi, model 150-20).

The formation and decay of porphyrin transients were also studied under steady state illumination at room temperature using an ESR-equipment (ER 200 D Bruker spectrometer), operating at 9.87 GHz, microwave power of 10 mW and magnetic field modulation of 3.2 G amplitude and 100 kHz frequency. Solutions containing 5×10^{-6} M HP or DHE in a flat quartz cell (0.2 ml, optical path 0.2 mm) were directly illuminated in the ESR cavity. The light source was a 300 W tungsten bulb, equipped with UV and IR cut-off filters. The light intensity on the cavity grid was determined to be 40 mW cm^{-2}. The photo-shutter, used for kinetic studies, allowed a time resolution of ca. 50 ms. Free radical concentrations were estimated using nitroxide radical (Tempone) as a standard.

Results and Discussion

a) Flash Photolysis Transient Spectra at pH 12.0

The HP-System

The ground state absorption spectrum of 3×10^{-6} M HP is shown as insert in Figure 2. The characteristic

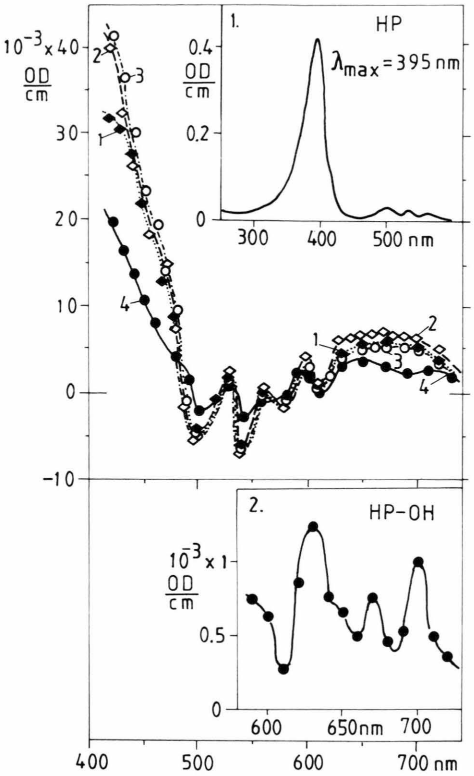


Fig. 2. Measured transient absorption spectra (50 μs after flash) of aqueous 3×10^{-6} M HP (pH 12) under various conditions. (1, ◆) airfree, (2, ◇) 2.8×10^{-2} M N_2O, (3, ○) 2.8×10^{-2} M N_2O, 0.1 M t-butanol and (4, ●) 2×10^{-2} M EDTA, 0.1 M t-butanol, 2.8×10^{-2} M N_2O (see text). – *Insert 1:* Ground state absorption spectrum of 3×10^{-6} M HP in aqueous solution at pH 12. *Insert 2:* Absorption spectrum of HP−OH species.

of the HP spectrum is typical for its non aggregated form.

The fate of the electronically excited HP molecules can be principally quite different

$$\mathrm{HP} \xrightarrow{h\nu} {}^1\mathrm{HP} \rightarrow e^-_{aq} + \mathrm{HP}^{\cdot +} \text{ (radical cation)} \quad (4a)$$
$$\rightarrow \mathrm{H} + \mathrm{HP}^{\cdot} \text{ (radical)} \quad (4b)$$
$$\rightarrow \mathrm{HP} + h\nu^1 \text{ (fluorescence)} \quad (4c)$$
$$\rightarrow {}^3\mathrm{HP} \rightarrow \text{chem. processes and phosphorescence.} \quad (4d)$$

In absence of oxygen the produced primary species can initiate a number of secondary reactions, e.g.:

$$e^-_{aq} + \mathrm{HP} \rightarrow \mathrm{HP}^{\cdot -} \text{ (radical anion)}, \qquad (5)$$
$$k_5 = 8.9 \times 10^9\ \mathrm{M}^{-1}\ \mathrm{s}^{-1} \quad [13].$$

Under the present conditions the H-atoms can be either transformed into e^-_{aq},

$$\mathrm{H} + \mathrm{OH}^-_{aq} \rightarrow e^-_{aq}, \qquad (6)$$
$$k_6 = 2.5 \times 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1} \quad [19]$$

or react with HP:

$$\mathrm{H} + \mathrm{HP} \rightarrow \mathrm{HP{-}H} \text{ (H-adduct)}. \qquad (7)$$

Assuming an upper value for $k_7 \leqq 1 \times 10^{10}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ it can be calculated that only less than 10% H will be consumed by reaction (7), whereby the rest is leading to the formation of $\mathrm{HP}^{\cdot -}$ via reactions (6) and (5).

The radical cation $\mathrm{HP}^{\cdot +}$ is very likely reacting with OH^- by electron transfer, resulting in OH radicals, which are scavenged by HP under formation of HP−OH adduct:

$$\mathrm{HP}^{\cdot +} + \mathrm{OH}^-_{aq} \rightarrow \mathrm{HP}^* + \mathrm{OH}, \qquad (8)$$
$$\mathrm{OH} + \mathrm{HP} \rightarrow \mathrm{HP{-}OH} \text{ (OH-adduct)}. \qquad (9)$$

The excited HP-molecules (HP*) resulting from the electron transfer process (8) have principally the same fate as the ^{1}HP species. Hence, in airfree HP solutions and in absence of OH-scavenger the formation of ^{3}HP, $\mathrm{HP}^{\cdot}$, $\mathrm{HP}^{\cdot -}$ and HP−OH will be expected. But, in the presence of t-butanol no OH-adduct will be formed; ^{3}HP, $\mathrm{HP}^{\cdot}$ and $\mathrm{HP}^{\cdot -}$ will be the transients.

In solutions saturated with N_2O, e^-_{aq} is converted into OH radical (reaction (2)), which can form HP−OH (reaction (9)) or be scavenged by t-butanol, when this is present in sufficient amount (reaction (3)). In the first case therefore, ^{3}HP, $\mathrm{HP}^{\cdot}$ and 2 HP−OH transients will be produced, whereas in the second one ^{3}HP and $\mathrm{HP}^{\cdot}$ species will be available.

The transient absorption spectra obtained by conventional flash photolysis of aqueous 3×10^{-6} M HP under various conditions at pH 12 (50 μs after flash) are presented in Figure 2. They show a very strong maximum in the range of 430–450 nm with a shoulder at about 470 nm. This absorption was previously assigned mainly to ^{3}HP state [7, 11, 12]. Based on the present experimental conditions it is obvious that the absorptions of $\mathrm{HP}^{\cdot}$, $\mathrm{HP}^{\cdot -}$ and HP−OH are superimposed with that of ^{3}HP in the investigated range of 400–750 nm. Spectrum 1 (^{3}HP, $\mathrm{HP}^{\cdot}$, $\mathrm{HP}^{\cdot -}$, HP−OH) obtained from airfree substrate solution differs significantly from that observed in the presence of N_2O (spectrum 2, Fig. 2; ^{3}HP, $\mathrm{HP}^{\cdot}$ and 2 × HP−OH). In the presence of t-butanol in a solution saturated with N_2O the sum of ^{3}HP and $\mathrm{HP}^{\cdot}$

species is observable (spectrum 3). By subtracting this spectrum from spectrum 2 the absorption spectrum of $2 \times HP-OH$ transients can be obtained. In insert 2, Fig. 2 the spectrum of HP–OH is presented. Since the OH radicals originate from different processes, the estimation of the HP–OH concentration and quantum yield is, however, difficult. The absorption range of HP–OH certainly covers the range from >300 to 750 nm with an ε-value higher than that of ^3HP in the range of 600–700 nm. The HP–OH fraction in spectrum 1 is obviously rather small, which explains the shift of spectrum 1 towards 2 in the range of 400–500 nm. In the same range ^3HP has a rather high ε-value.

It is well known that EDTA is acting as an efficient electron donor and is able to reduce ^3HP (conversion to $HP^{\cdot -}$). By flashing a solution of 3×10^{-6} M HP in the presence of 2×10^{-2} M EDTA, 0.1 M t-butanol saturated with N_2O, the absorption spectrum of $HP^{\cdot -}$ superimposed with the rest of ^3HP ($\sim 55\%$) is obtained,

$$^3HP + EDTA \rightarrow HP^{\cdot -} + EDTA^{\cdot +}. \qquad (10)$$

The measured absorption spectrum $HP^{\cdot -}$ with ^3HP is shown in Fig. 2, spectrum 4. Since the ε-value of ^3HP in the range of 550 to 750 nm is rather small, the observed absorption belongs mostly to the $HP^{\cdot -}$ species.

The HPD-System

The ground state absorption spectrum of aqueous 1.5×10^{-6} M HPD at pH 12 is presented in the insert of Figure 3. Its Soret band exhibits a maximum at 374 nm with slight shoulder at about 395 nm. The broad absorption band indicates that the substrate is present partly in aggregated form.

Under illumination of HPD the same photoinduced processes can occur as shown for HP (reactions (4) to (9)). The transient absorption spectra obtained by flashing 1.5×10^{-6} HPD at pH 12 in the presence of various additives are given in Figure 3. The spectrum 1, resulting from airfree substrate, represents a superimposed absorption of ^3HPD, $HP^{\cdot}$, $HPD^{\cdot -}$ and HPD–OH. In the presence of N_2O, e_{aq}^- is converted into OH and hence spectrum 2 contains ^3HPD, $HP^{\cdot}$ and $2 \times HPD-OH$ species.

By addition of 0.1 M t-butanol, which scavenges efficiently the OH radicals, the corresponding spectra 3 (^3HPD, $HPD^{\cdot}$, $HPD^{\cdot -}$) and 4 (^3HPD, $HPD^{\cdot}$) are observed. By subtracting spectrum 4 from 3 the absorption of $HPD^{\cdot -}$ is obtained (Fig. 3, insert 2). This transient has a rather low absorption in the range of 500 to ca. 800 nm with maxima at 600 and about 700 nm. Obviously the yield of $HPD^{\cdot -}$ under the present conditions is rather low. In the range 400 to 500 nm the ^3HPD absorption predominates. In addition to this also the spectrum of HPD–OH radicals is obtained as the difference of spectra 1 and 3 (see Fig. 3, insert 2). Its main maximum is about 700 nm. Again, this transient is formed also in rather small concentration compared to the triplet state. It should be mentioned that the edition of EDTA did not affect the OD-values of the spectra in abscence of the reducing agent.

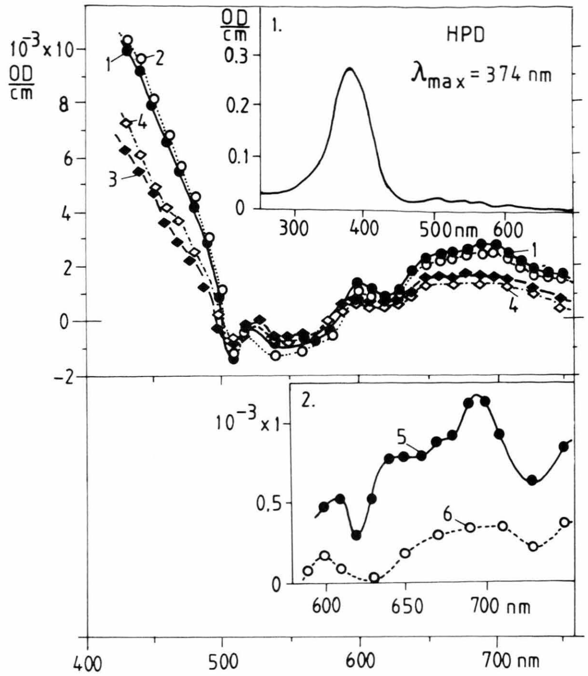


Fig. 3. Measured absorption spectra (50 µs after flash) of transients produced from 1.5×10^{-6} M HPD under various conditions: (1, ●) airfree, (2, ○) 2.8×10^{-2} M N_2O, (3, ◆) airfree, 0.1 M t-butanol and (4, ◇) 2.8×10^{-2} M N_2O, 0.1 M t-butanol. – *Insert 1:* Ground absorption spectrum of 1.5×10^{-6} M HPD in aqueous solution at pH 12. *Insert 2:* Absorption spectrum of HPD–OH (5) and $HPD^{\cdot -}$ (6).

b) Flash Photolysis Transient Spectra at pH 7.4 in Homogeneous and Microheterogeneous Environment

HP/Additives System

The ground state absorption spectra of 3×10^{-6} M HP in phosphate buffer solution (pH 7.4) is typical for aggregated free base porphyrin substrates, while in the presence of a neutral detergent "Triton X 100" the porphyrin undergoes disaggregation (Fig. 4, insert). The transient absorption spectra of airfree aqueous 3×10^{-6} M HP in the presence of t-butanol in phosphate buffer (pH 7.4) without and in the presence of various additives are shown in Figure 4. Generally, the shape of the spectra is rather similar to that for the same substrate, observed at pH 12 (Fig. 2), but in the present case the OD-values are much lower. This fact is attributed to the increase of the triplet quenching related to the higher aggregation state in neutral solution (compare spectrum 1, Fig. 2, with that in Figure 4). In the presence of EDTA in airfree substrate solution the absorption is lowered considerably (spectrum 2, Fig. 4), because part of the triplet is reduced by EDTA, according to reaction (10).

In the presence of Triton X 100 a strong decrease of the whole absorption spectrum occurs (spectrum 3, Figure 4). This is in contradiction of the expected rising of the absorption as a consequence of the disaggregation of the substrate molecules by the detergent. Hence, the observed effect is most likely explainable by a quenching of ^{3}HP due to the hydrocarbon chains of the micelles. In addition to this, the residual oxygen in the micelles can also contribute to the effect. Adding 2×10^{-2} M EDTA as reducing agent to the substrate (reaction (10)) an increase of the absorption due to $HP^{\cdot -}$ formation is observed (spectrum 4, Figure 4). Further, as well known in the presence of Triton X 100, the diffusion of the radicals is strongly suppressed. This was also established by comparison of the second order decay rates in the absence and presence of micelles.

Based on data given in Figs 2 and 3 and on literature data concerning the triplet of various porphyrines it is to be stressed that the absorption band in the range 400–550 nm in Fig. 4 is mostly due to the ^{3}HP. The rest of the absorption (500–750 nm) represents in each case a superimposed absorption of various transients and a rather small contribution by ^{3}HP.

Having in mind that ^{3}HP is transformed at least partly into $HP^{\cdot -}$ in the presence of EDTA, on the basis of the present observations one can assume that the substrate is located near the polar interface of the micelles.

In neutral HP solutions principally a mixture of various transients (^{3}HP, $HP^{\cdot -}$, $HO^{\cdot +}$, $HP^{\cdot}$, HP–H) is formed under the influence of light. Hence it is very difficult to evaluate their individual spectroscopic and kinetic characteristics. However, by subtracting spectrum 3 from 4 the absorption spectrum of $HP^{\cdot -}$ in neutral aqueous solution is obtained (insert 2, Figure 4).

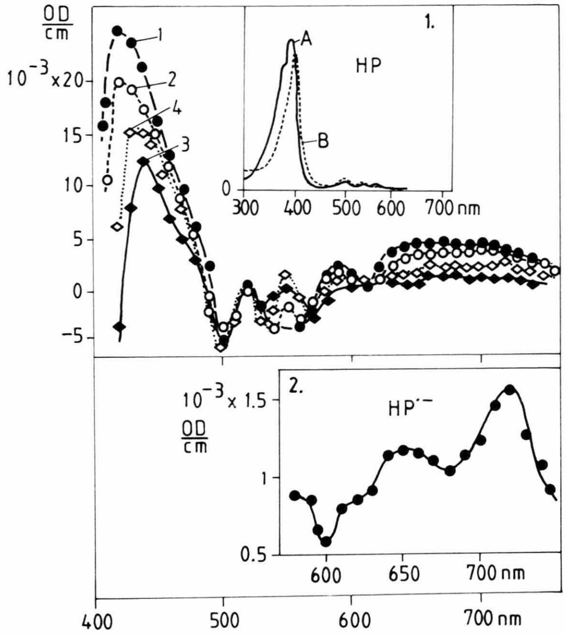


Fig. 4. Measured transient absorption spectra resulting from aqueous airfree 3×10^{-6} M Hp in the presence of 0.1 M t-butanol and phosphate buffer (pH 7.4): (1) no additives, (2) 2×10^{-2} M EDTA, (3) 2% v/v Triton X 100 micelles, (4) 2×10^{-2} M EDTA and 2% v/v Triton X 100 micelles. – *Insert 1:* Ground state absorption spectra of aqueous HP in: (A) phosphate buffer (pH 7.4) and (B) Triton X 100 micelles. *Insert 2:* Absorption spectrum of $HP^{\cdot -}$ transients (see text).

HPD/Additives System

Flash photolysis experiments of aqueous HPD at pH 7.4 in the presence of t-butanol under conditions similar to those described above, were performed. The ground state absorption spectrum of HPD at pH 7.4 (phosphate buffer) indicates the high aggregate form of the substrate. By adding Triton X 100 micelles obviously a strong disaggregation effect takes place (insert 1, Figure 5). Spectrum 1 (Fig. 5), obtained in airfree HPD (pH 7.4; 0.1 M t-butanol) solutions shows a rather low triplet absorption in the range of 410–500 nm. This is as expected due to the strong aggregated substrate form. The observed second order mixed kinetics in this range is rather fast, hence the measured spectra at 50 μs after flash represent only a

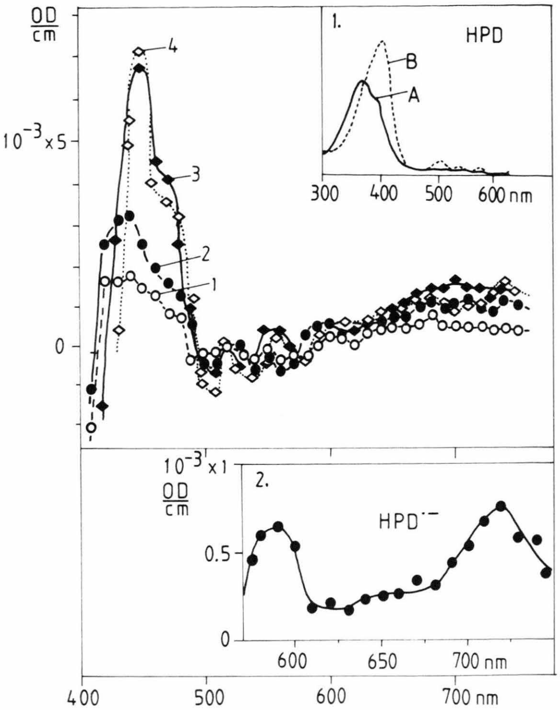


Fig. 5. Measured transient absorption spectra obtained from aqueous airfree 1.5×10^{-6} M HPD in the presence of 0.1 M t-butanol and phosphate buffer (pH 7.4): (1) no additives, (2) 2×10^{-2} M EDTA, (3) 2% v/v Triton X 100 micelles and (4) 2×10^{-2} M EDTA, 2% v/v Triton X 100 micelles. – *Insert 1:* Ground state absorption spectra of aqueous HPD in: (A) phosphate buffer (pH 7.4) and (B) Triton X 100 micelles. *Insert 2:* Absorption spectrum of $HPD^{\cdot -}$ (see text).

part of their initial values. When 2×10^{-2} M EDTA is added to the HPD-solution an essential increase of the absorption is established, which is mainly attributed to the formation of $HPD^{\cdot -}$ (spectrum 2, Figure 5). Spectrum 3 (Fig. 5) represents in the first place the increased yield of ^{3}HPD, as a consequence of the substrate disaggregation by the added micelles. Simultaneously to this effect also a quenching of ^{3}HPD takes place, which is based on the special micellar character mentioned above in the case of HP (Figure 4). However, the competition between these two processes in the present case is in favour of the ^{3}HPD formation.

The addition of 2×10^{-2} M EDTA to the HPD/Triton X 100 system does not change significantly the transient absorption spectrum, although an appreciable amount of $HPD^{\cdot -}$ (reaction (10)) should be formed (spectrum 4, Figure 5). The reason for that might be the deeper immersion of HPD within the micellar core, which can cause a restricted accessibility of EDTA. The spectrum of $HPD^{\cdot -}$ species (Fig. 5, insert 2) is obtained by subtracting the spectra 1 from 2.

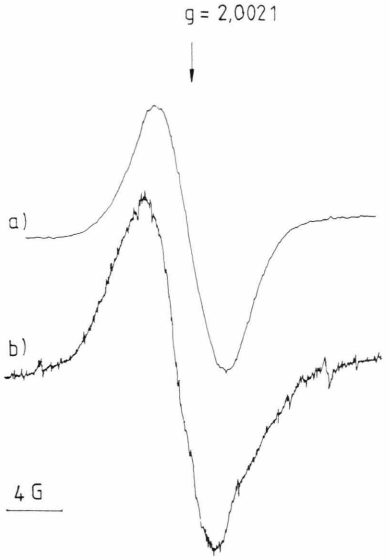


Fig. 6. ESR spectra of species obtained under steady-state illumination at room temperature of airfree 5×10^{-4} M HP, at pH 7.4 (phosphate buffer) in the presence of 2×10^{-2} M EDTA (a) and of aerated 5×10^{-4} M HPD (pH 7.4) (b). Conditions: microwave power 10 mW, microwave frequency 9.87 GHz, field modulation 100 kHz, amplitude 3.2 G, Gain a) $\times 10^5$ and b) $\times 10^6$.

Finally, it might be mentioned that in buffer solution the yield of the radicals is expected to be higher than that of ^{3}HPD.

c) Photoinduced HP and HPD Transients Studied by ESR Technique

The formation and decay of free radicals produced by steady state light irradiation of aqueous HP and HPD solutions at pH 7.4 (phosphate buffer) under various conditions were studied by ESR spectroscopy. The irradiation of airfree 5×10^{-4} M HP (aggregated form) in the presence of 2×10^{-2} M EDTA resulted in a well detectable single line (gaussian in shape) ESR signal, without any hyperfine structure (Fig. 6, spectrum a; Table 1). In the absence of EDTA under otherwise similar conditions, however, no signals could be observed. Based on this fact, and on the data discussed in the previous two sections, it seems very likely that in the first case (presence of EDTA) the observed ESR signal corresponds to the $HP^{\cdot -}$ transient, which is resulting from reaction (10). This result is in accor-

Table 1. Free radicals resulting from HP and DHE under different conditions, observed by ESR-technique.

Parameters	g	$W(G)$	k_2 (M^{-1} s^{-1})	Steady state radical concentr. (M)
HP (pH 7.4 Ar + EDTA)	2.0021	5.0	$> 1.0 \times 10^6$	1.0×10^{-5}
HP (pH 7.4, Ar)	–	–	–	–
HP (pH 4.0 to 7.4, air)	2.0023	4.6	1.5×10^5	1.0×10^{-6}
DHE (pH 7.4, Ar + EDTA)	–	–	–	–
DHE (pH 7.4, Ar)	2.0023	4.3	1.7×10^4	1.1×10^{-6}
DHE (pH 7.4, air)	2.0024	4.3	9.3×10^3 3.6×10^4	1.5×10^{-6}
DHE (pH 7.4, air + Triton)	2.0025	4.4	1.2×10^5	1.0×10^{-6}

Porphyrin concentration 5×10^{-4} M, EDTA concentration 2×10^{-2} and Triton X 100 2% v/v; $W(G)$ peak-to-peak line width in Gauss.

dance with the porphyrin reduction and formation of phlorin as previously proposed by Mauzerall and Feher [15, 20]. Further, it is obvious that the concentration of all other above discussed radicals (HP – H, HP – OH and HP$^{\cdot}$) is very low and not detectable under the applied experimental conditions.

In aerated 5×10^{-4} M HP solution (pH 4–7) appears a weak, but relatively long-lived transient ($\tau / \sim 1.2$ s) with spectrum parameters similar to those of HP$^{\cdot -}$ (Table 1). The obtained spectrum seems to be a superimposed one, consisting of HP-peroxy-radicals (HP – OO$^{\cdot}$) and HP$^{\cdot +}$. In this process, the generated singlet oxygen ($^1O_2$) as a sequence of the photoinduced reaction (4d) may play an important role, too. It is noteworthy that under the same conditions not any transients were registrated by flash photolysis. This is probably because the peroxy-radicals have low light absorbance, and as can be seen from Table 1, their steady state concentration is rather low.

The behaviour of HPD under steady-state illumination, studied by ESR technique, was found to differ from that of HP ones. In airfree, EDTA containing solution of HPD no detectable transients were recorded. Two possibilities for the explanation of this fact can be taken into account: i) The electron delocalization within the covalently linked porphyrin rings can result in a very broad (undetectable) ESR signal of HPD$^{\cdot -}$. ii) Due to the lower amount of side chain charged groups a fast recombination (back reaction) of HPD$^{\cdot -}$ with EDTA$^{\cdot +}$ could take place. In the absence of EDTA (airfree solution), however, a weak long lived free radical was detected, which could be attributed to HPD$^{\cdot +}$ and/or HPD$^{\cdot}$ species, which is expected to be long lived under this conditions.

In the presence of oxygen, or in aerated Triton X 100/HPD system, free radicals were observed, too (see Fig. 6, spectrum b and Table 1). In this case the line shape is inhomogeneous, indicating the presence of more than one transient. It is likely that in buffer solution both HPD$^{\cdot +}$ and HPD – OO$^{\cdot}$ species are simultaneously formed, which is indicated by the presence of two different second order decay rates (Table 1). In solutions containing Triton X 100 micelles the formation of HPD – OO$^{\cdot}$ is less likely, due to the diffusion limitation of the transients. The increased local concentration of HPD molecules in the micelle core can lead to an enhanced decay rate (probably of HPD$^{\cdot +}$ and/or HPD$^{\cdot}$, Table 1). It is also notable that the HPD aggregate breakdown by Triton X 100 micellization does not significantly alter the free radical yield (the steady state concentration remains in the range of $1.0–1.5 \times 10^{-6}$ M, Table 1). The later result should imply that intermolecular interactions (between HPD aggregates) are not involved in the charge transfer reactions responsible for the HPD free radical formation.

Conclusion

As known, various hematoporphyrin derivatives were successfully applied for photodynamic treatment of tumors in man. In order to learn more about the primary photoinduced processes and the involved transients, comparative investigations were undertaken with HP and HPD (photofrin II) in the presence of various additives, making use of conventional flash photolysis and ESR technique.

The observed transient absorption spectra in the range of 420–460 nm at pH 12 represent in the first place the triplet excited state (^{3}HP and ^{3}HPD), which is superimposed with the absorption bands of various free radicals (HP$^{\cdot}$, HP$^{\cdot -}$, HP – OH and HPD$^{\cdot}$, HPD$^{\cdot -}$, HPD – OH, respectively). The relatively weak plateau-shaped transient absorption from about 500 to 750 nm is assigned predominantly to the above free radicals. Using N_2O and t-butanol as specific scavengers for e_{aq}^- and OH species, respectively and by appropriate subtraction of the superimposed absorptions, the spectra of HP–OH, $HPD^{\cdot -}$ and HPD–OH transients were obtained. EDTA was found to be a rather strong reducing agent for ^{3}HP and ^{3}HPD, converting them efficiently (40–50%) into $HP^{\cdot -}$ and $HPD^{\cdot -}$.

In neutral HP and HPD solutions (pH 7.4, phosphate buffer) both substrates are in aggregated form. However, in the presence of Triton X100 micelles disaggregation takes place. As a rule, the HPD-transients decay more rapidly than those resulting from HP. EDTA can efficiently quench ^{3}HP, when the micelle concentration is sufficient to promote a disaggregation of the substrate. Here again ^{3}HP and ^{3}HPD have a strong absorption band in the range of 420–440 nm and a weak absorption from 500 to 750 nm, where various transients resulting from both substrates possess their absorption bands. By appropriate subtraction of spectra the absorption bands of $HP^{\cdot -}$ and $HPD^{\cdot -}$ at pH 7.4 were obtained.

The ESR studies proved that several photoinduced transients are formed with small yields. In the presence of EDTA the irradiated airfree HP resulted in a well pronounced ESR signal, which was attributed to the $HP^{\cdot -}$ transient. In aerated HP-solutions (pH 4–7) singlet oxygen ($^1O_2$) and peroxyradicals seem to be the important species in the system.

In contrast to HP, the HPD/EDTA system did not lead to any detectable species. In absence of EDTA the airfree HPD-solution resulted in a weak but long-lived radical, which was asssigned to $HPD^{\cdot +}$ and/or $HPD^{\cdot}$. Applying Triton X100 as disaggregation auxiliary for HPD at pH 7.4 in the presence of oxygen superimposed ESR spectra were obtained, which result most likely $HPD^{\cdot +}$ and $HPD{-}OO^{\cdot}$.

On the base of the obtained experimental data it is assumed that the short-lived transients detected under flash photolysis conditions are the precursors of the relatively long-lived species observed by ESR-technique. Very likely the second ones are involved in secondary photochemical processes. The various transients discussed here might play an important role in the photodynamic treatment of tumors in man.

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