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Kinetic studies on the mechanism of haematoporphyrin derivative photobleaching

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Abstract. Haematoporphyrin derivative (HPD), a sensitizer used in photodynamic therapy (PDT) of tumours, is progressively destroyed (photobleached) during illumination. However, the mechanism of the sensitizer photobleaching remains unclear, although its degradation presents both potential problems and potential advantages. This paper surveys the effects of reaction conditions, photooxidizable biomolecules, electron acceptors, and other agents on the quantum yield (QY) and kinetics of the photodestruction of HPD in solution. The initial QY of HPD photobleaching in pH 7.4 phosphate buffer in air was measured as 3.6×10^{-5}. The yield decreased significantly in organic solvents with a low dielectric constant and in the presence of various surfactants. Ionic strength, pH, and temperature had relatively slight effects on the photobleaching yield of HPD. For example, raising the temperature from 10 to 43 °C caused only a moderate (about 2-fold) increase in the QY of HPD photobleaching. It was found that oxygen is needed for the photobleaching of HPD. However, the QY of HPD photodestruction increased only slightly (by 55%) in D_2O. Sodium azide, an efficient physical quencher of singlet oxygen ($^1O_2$), had only a slight effect on the photobleaching yield, even at 50 mM. Our data suggest that besides $^1O_2$, free radical reactions are involved in the photodegradation of HPD in aqueous solution. In fact, the QY of HPD photobleaching decreased in the presence of hydroxyl radical scavengers, such as mannitol, sodium benzoate, ethanol, and deferoxamine. In addition, the photodestruction of HPD could be associated with the formation of very reactive cation radicals of the sensitizer. Some photooxidizable substrates and model electron acceptors increased markedly the photobleaching efficiency of HPD. At certain concentrations, the QY of HPD photobleaching was enhanced by the presence of histidine, reduced glutathione, dithiothreitol, and lecithin. Electron acceptors, such as metronidazole and flavin mononucleotide, also increased the photobleaching yield. In contrast, NADH and cysteine, which are electron donors, inhibited the rate of HPD photodestruction. High concentrations (>1 mM) of biological antioxidants, such as ascorbic acid and α-tocopherol, also increased the photostability of HPD in solution. These results suggest that the mechanism(s) of the photobleaching of HPD in cells and tissues during PDT may be complex.

Key words: haematoporphyrin derivative, photobleaching, photodynamic therapy, tumour.

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Abbreviations: ASA = ascorbic acid; CAT = catalase; CTAB = cetyltrimethylammonium bromide; DMFA = N,N-dimethylformamide; D_2O = heavy water; DEF = deferoxamine mesylate; FMN = flavin mononucleotide; HP = haematoporphyrin; HPD = haematoporphyrin derivative; $HPD^{+\bullet}$ = HPD cation radical; MB = methylene blue; NaN_3 = sodium azide; $^1O_2$ = singlet oxygen; $O_2^{-\bullet}$ = superoxide anion radical; $OH^\bullet$ = hydroxyl radical; PDT = photodynamic therapy; PS = photosensitizer; PII = Photofrin II; SPB = sodium phosphate buffer; SOD = superoxide dismutase; SDS = sodium dodecyl sulphate; α-TOC = α-tocopherol; Trp = L-tryptophan; TX-100 = Triton X-100.

INTRODUCTION

On illumination, many porphyrins in simple solution are photobleached, i.e. they are converted into products that do not absorb appreciably in the visible range of spectrum. In this process, the porphyrin macrocycle is usually disrupted, probably by the attack of singlet oxygen ($^1O_2$) generated by the photoexcited porphyrin, although free radical reactions may also occur [1]. In general, the organic chemistry of the photobleaching of free base porphyrins is not well understood; more research has been done on the photodestruction of metallo-porphyrins [2–4]. At present, photochemical transformations of porphyrins (both of natural origin and especially synthesized) are the subject of intensive studies [5, 6] because these compounds are applied as sensitizers in photo-dynamic therapy (PDT) of tumours [7].

Haematoporphyrin derivative (HPD) and its improved version, known as Photofrin II (PII), are the most widely used photosensitizers (PS) in PDT of tumours. HPD is a complex mixture of porphyrins prepared from haemato-porphyrin (HP) by acetylation and subsequent alkaline hydrolysis. Typically this mixture consists of approximately 20% HP, 20–30% hydroxy-ethyl-vinyl-deuteroporphyrin, and 3–5% protoporphyrin. The other half, which is responsible for the antitumour effect of PDT in vivo, is a mixture of dimers, trimers, and some higher molecular weight oligomers (up to eight porphyrin units) [8]. The porphyrin units are linked through ester and ether bonds, but a C–C linkage has also been suggested [9]. PII, in comparison to HPD, is a porphyrin mixture enriched in the above-mentioned oligomers.

Until recently, it was assumed that HPD and PII are photochemically stable during the treatment and can be activated indefinitely to produce the desired therapeutic effect. Moan et al. [10, 11] were the first to demonstrate the possible photooxidation of HPD in cells treated in culture. Similar photobleaching of PII has been observed in tumours of animals [12, 13] and in patients undergoing PDT [12]. In principle, the degradation of HPD or PII during PDT presents both potential problems and potential advantages. If these sensitizers are bleached too rapidly during photoirradiation, the tumour may not be destroyed completely [10, 12]. Therefore, the photodegradation of HPD has an effect on the PDT clinical dosimetry [5, 12]. On the other hand, it has been suggested [14] that photobleaching could be used as a means to eliminate skin photosensitivity, the main side effect observed in patients undergoing HPD-based PDT.

During the past decade, it was also established that HPD and PII are photodegraded more rapidly in tumour cells than in saline [15, 16]. However, the exact molecular mechanisms of the phenomenon remain unclear.

Thus, it is of interest to examine the photobleaching behaviour of porphyrins proposed for use in PDT of tumours. This paper surveys the effects of reaction conditions, photooxidizable biomolecules, electron acceptors, and other agents on the quantum yield and kinetics of the photodestruction of HPD in solution.

MATERIALS AND METHODS

Chemicals

HPD was synthesized from haematoporphyrin IX dihydrochloride (Aldrich) according to the original method of Lipson et al. [17] modified by Kessel et al. [18]. The obtained product was diluted with twice distilled water to a final porphyrin concentration of 5 mg/mL, and stored in the dark at −70 °C (the solution pH was 7.4). Working solutions of HPD were made using the same arbitrary molecular weight as for HP (598.71). Sodium azide (NaN_3), heavy water (D_2O), lecithin (from egg yolk), deferoxamine mesylate (DEF), flavin mononucleotide sodium salt (FMN), superoxide dismutase (SOD; from bovine erythrocytes, activity 3300 units/mg), catalase (CAT; from bovine liver, activity 10900 units/mg protein), and all other chemicals (of analytical grade or better) were purchased from Sigma, St. Louis, USA.

Photobleaching measurements

In a typical experiment, an 8 mL sample of a reaction mixture (containing HPD without or jointly with other compounds) was placed in a 20×20 mm quartz cuvette and a microstirring magnet was added. The cuvette was then placed in a thermostatted (by circulating water) holder and illuminated in air with stirring, using a voltage regulated 1 kW xenon arc-lamp provided with a glass filter (FS-1) that transmits 80% of light at 395 nm (the range between 330–470 nm). The incident light fluence rate was 124 mW/cm^2, as measured by an IMO-2N radiometer (Russian Federation). The fall of HPD absorbance in the Soret peak (around 400 nm) was measured to determine the rate of its photodegradation. Absorption spectra of HPD solutions were recorded by means of a Specord M-40 spectrophotometer (Germany). Quantum yields of HPD photobleaching were calculated as the ratio of the initial rate of disappearance of the porphyrin molecules to the initial rate of absorption of photons by the reaction mixture. The fraction of light energy absorbed by the reaction mixture was determined with a ferrioxalate actinometer [19]. Unless otherwise indicated, reaction mixtures were 16.7 μM in HPD, 100 mM in sodium phosphate buffer (SPB) of pH 7.4 and 0.28 mM in oxygen (air-equilibrated). The temperature was 20 ± 1 °C. Samples were bubbled with tank nitrogen for 30 min for the low oxygen concentration experiments.

Other measurements

The quantum yields of HPD-sensitized photodestruction of L-tryptophan (Trp), defined as the ratio of the number of molecules of photooxidized Trp to the number of photons absorbed by HPD, were usually measured at 20 °C. Under photoexcitation of HPD, the absorbance (differential spectrum) of Trp at 280 nm was registered to determine the rate of its photooxidation. Unless otherwise indicated, reaction mixtures were 16.7 μM in HPD, 100 mM in SPB of pH 7.4, and 0.2 mM in Trp. Other experimental conditions (light source, intensity of the emitted light at 395 nm, irradiation procedure, etc.) were the same as described in the section "Photobleaching measurements".

Statistics

Standard errors in the measurements of quantum yields were (for HPD and Trp) in the ±5–7% range, as estimated from three independent experiments.

RESULTS AND DISCUSSION

Time course and quantum yields of HPD photobleaching in water: effects of oxygen concentration, NaN_3, and D_2O

In aqueous solution, the time course of HPD photobleaching was a mixed order process, suggesting that some components of the sensitizer are more sensitive to photodegradation than others, as has been observed during the laser photobleaching of PII [15]. In air-equilibrated 100 mM SPB of pH 7.4, the quantum yield of HPD photobleaching was measured as 3.61×10^{-5}. Results similar to those for HPD were obtained by others [20] (in the same buffer, but at 25 °C) with PII (5.4×10^{-5}) and typical porphyrins (HP, 4.7×10^{-5}; uroporphyrin, 2.8×10^{-5}). Moreover, HPD and PII, in comparison with other PSs proposed for the use in PDT of tumours, show an increased light stability. For instance, in aqueous buffer the quantum yield of photobleaching of L-aspartyl chlorin-e_6 (8.2×10^{-4}) [6] exceeds that for HPD almost 23-fold.

Some porphyrins in simple solution appear to be photodegraded in reactions induced by self-generated $^1O_2$ [3]. On illumination, HPD produces $^1O_2$ with a quantum yield of 0.64 [21]. Therefore, the photobleaching of the PS may be mediated by this oxygen species. In order to evaluate the role of $^1O_2$, we examined the effects of oxygen concentration, NaN_3, and D_2O on the rate of HPD photodegradation. It was found that under nitrogen the initial quantum yield of HPD photobleaching was reduced to 39% of that in air (Table 1). Thus, oxygen is needed for the photobleaching process.

Table 1. Quantum yields of HPD (16.7 μM) photobleaching under different conditions relative to the control value (3.61×10^{-5} molecules per absorbed photon) in air-equilibrated 0.1 M SPB (pH 7.4) at 20 °C

Conditions	Relative quantum yield of photobleaching
Control	1.0
30 min nitrogen bubbling before irradiation	0.39
NaN_3 (10 mM)	0.95
NaN_3 (50 mM)	0.87
0.1 M SPB (pH 7.4) prepared with D_2O	1.55

NaN_3 is an efficient physical quencher of $^1O_2$ ($k_q = 5.8 \times 10^8$ M^{-1} s^{-1}) [22]. Hence, this agent must inhibit the rate of the sensitized photooxidation of substrates under conditions where the $^1O_2$ concentration is rate limiting. To our surprise, NaN_3, even at the highest concentration used (50 mM), had little effect on the quantum yield of HPD photobleaching, reducing it only by 13% (Table 1); at this concentration, NaN_3 almost completely inhibited the HPD-photosensitized oxidation of 0.2 mM Trp that is mediated by $^1O_2$ [23] (data not shown). However, this may not rule out the involvement of $^1O_2$ in the photobleaching process because $^1O_2$, as known, is generated very close to the excited molecule of HPD; as a result, the $^1O_2$ molecule has a much higher probability of reacting with the HPD molecule that generated it than with NaN_3 in solution.

One of the most reliable methods to prove the participation of $^1O_2$ in photosensitized reactions is using D_2O as a solvent. The method is based on almost 15-times longer lifetime of $^1O_2$ in D_2O as compared to that in H_2O [24]. Nevertheless, the quantum yield for HPD photobleaching was only 55% greater in D_2O than in H_2O (Table 1). Thus, even though oxygen is required for the photodegradation of HPD, the $^1O_2$ lifetime over the H_2O–D_2O range does not appear to be an important limiting factor in the photobleaching of the porphyrin under the reaction conditions used.

On the basis of the experiments performed with NaN_3 and D_2O it is not clear what role $^1O_2$ may play in the photobleaching of HPD.

Effects of organic solvents on HPD photobleaching yields

For these measurements, aliquots of the stock (5 mg/mL) HPD solution were pipetted into test tubes, evaporated down under a vacuum, redissolved in corresponding solvents, and the photobleaching quantum yields were determined. The results obtained show that the yields varied over a large range in the chosen solvents (Table 2). The bleaching yields in organic solvents were smaller than those in aqueous buffer, except in formamide, where the quantum yield of HPD photodegradation was slightly larger than in buffer. Table 2 lists three properties of the solvents that might be expected to have an effect on the photobleaching yields: $^1O_2$ lifetime, O_2 concentration (in equilibrium with air), and dielectric constants. There is no apparent correlation between $^1O_2$ lifetime and oxygen

Table 2. Quantum yields of the photobleaching of 16.7 μM HPD in air-equilibrated aqueous buffer (100 mM SPB, pH 7.4) and organic solvents at 20 °C

Solvent	Quantum yield	Properties of the solvents [6, 24]		
		$^1O_2$ lifetime, μs	O_2 concentration, mM	Dielectric constant
Aqueous buffer	3.61×10^{-5}	3.1–4.2	0.28	78
Ethanol	1.57×10^{-6}	9.7–15.3	2.07	24.3
Methanol	4.05×10^{-7}	5–12	2.12	32.6
Dimethylformamide	1.50×10^{-5}	7	1.0	36.7
Formamide	3.75×10^{-5}	6.7	–	84–109

solubility in the solvents and the quantum yields of HPD photobleaching. In fact, the $^1O_2$ lifetime is larger in ethanol than in water; however, the bleaching yield for HPD is very low in ethanol. The oxygen concentrations in the alcohols and N,N-dimethylformamide (DMFA) are greater than in water, but the quantum yields of HPD photobleaching are much lower than in the aqueous buffer.

There is, however, a crude correlation between the quantum yields of HPD photodestruction and the dielectric constants of the solvents. DMFA and the alcohols (methanol, ethanol) have lower dielectric constants than water, and the yields were lower in all these solvents than in aqueous buffer (Table 2). It was also established that a decrease in the medium polarity (upon addition of ethanol or DMFA to aqueous solution of HPD) strongly inhibited the efficiency of the sensitizer photodegradation (Fig. 1), although the increase of ethanol (or DMFA) concentration in reaction mixtures may enhance the production of $^1O_2$ by HPD [21]. Similar effects of organic solvents on the photobleaching behaviour of

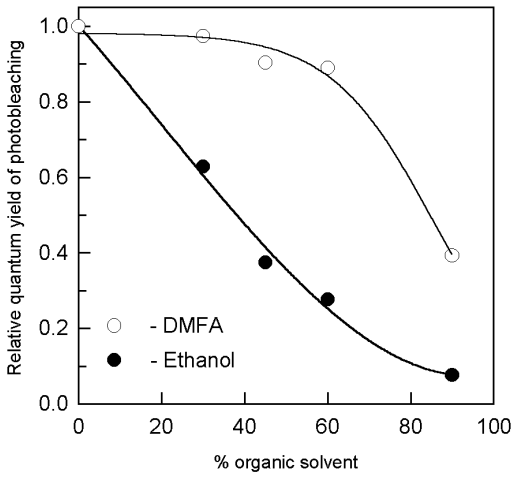

Fig. 1. The effect of the percentage of organic solvent on the quantum yields of HPD (16.7 μM) photobleaching in various water (pH 7.4-buffered solutions)–organic solvent mixtures relative to the value (3.42×10^{-5}) in 0.2 mM SPB (pH 7.4) at 20 °C.

some porphyrins were also registered in other laboratories. For example, Reddi et al. [25] reported that tin-protoporphyrin is much more light stable in methanol than in water. However, the mechanism(s) that might be participating in this general correlation involving solvent dielectric properties is (are) not clear. Several possible explanations can be offered for the results about the effect of medium polarity on photostability of porphyrins. It was demonstrated that the reactivity of photogenerated $^1O_2$ toward a substrate may depress in solvent mixtures less polar than water [23]. On the other hand, H_2O, as a solvent having a high dielectric constant, can promote the photoprocesses with charge transfer and, as a consequence, enhance the formation of ionic intermediate species and porphyrin destroying free radicals.

Effects of CAT, SOD, and hydroxyl radical traps on HPD photobleaching

It has been demonstrated that superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) can be produced in addition to $^1O_2$ by some PSs upon illumination [26, 27]. They are reactive forms of oxygen and might mediate HPD photodegradation. This is highly probable since in the previous work [16] we showed that the PS is very reactive towards $OH^\bullet$. To determine the role of free radicals, the effects of specific antioxidants on the quantum yield of HPD photobleaching were investigated.

It is commonly accepted that SOD protects against free radical injury by converting $O_2^{-\bullet}$ to H_2O_2, provided that H_2O_2 can be removed by CAT, which catalyzes the decomposition of H_2O_2 to water and oxygen [28]. Consequently, impeding the coexistence of $O_2^{-\bullet}$ and H_2O_2 would prevent $OH^\bullet$ formation through the $O_2^{-\bullet}$-driven Fenton reactions [24] and thereby increase the photostability of HPD. The effects of CAT and SOD on the light resistance of the PS were surprising. Indeed, the quantum yield of HPD photobleaching was unchanged on addition of CAT, but substantially (over 2-fold) increased in the presence of SOD (Table 3). This effect of SOD could be associated with an increased production of H_2O_2. However, the addition of CAT (up to 0.1 mg/mL) did not abolish the increasing effect of SOD on the rate of HPD photobleaching (Table 3). We believe that the catalyzing effect of SOD was largely mediated by stimulation of the very reactive HPD cation radical ($HPD^{+\bullet}$) formation (the enzyme, as known, suppresses the bimolecular reaction of porphyrin cation radicals with $O_2^{-\bullet}$ [29]). It is important to note that the formation of $HPD^{+\bullet}$ in water solutions was registered in other laboratories by flash photolysis and ESR studies [30]. Thus, our findings suggest that in aqueous solution and in the presence of molecular O_2 (an electron acceptor) the photodegradation of HPD could be associated with its one-electron oxidation leading to the formation of $HPD^{+\bullet}$. As known, the charge of these species is rapidly lost by a number of processes, including hydration (thereby yielding hydroxylated products) [31]. Furthermore, $HPD^{+\bullet}$ can potentially react with oxygen to give oxidized products.

Table 3. Quantum yields of HPD (16.7 μM) photobleaching under different conditions relative to the yield in air-equilibrated 100 mM SPB (pH 7.4) at 20 °C (the control quantum yield of photobleaching is given in Table 2)

Conditions	Relative quantum yield of photobleaching
Control (no additive)	1.0
Catalase (up to 0.1 mg/mL)	1.01
Superoxide dismutase (up to 0.1 mg/mL)	2.07
Superoxide dismutase plus catalase	2.1
Mannitol (up to 100 mM)	0.9
Sodium benzoate (up to 100 mM)	0.87
Ethanol (up to 100 mM)	0.81
Deferoxamine mesylate (up to 0.01 mM)	0.42

We also examined the effects of several effective traps of $OH^{\bullet}$ (such as mannitol [32], sodium benzoate [33], and ethanol [33]) on HPD photodegradation. According to Table 3, the quantum yields of HPD photobleaching decreased: 10% for mannitol (at 0.1 M), 13% for sodium benzoate (0.1 M), and almost 20% for ethanol (0.1 M). This indicates that $OH^{\bullet}$ radicals are involved in the photobleaching of HPD. To determine whether $OH^{\bullet}$ generation proceeds via the reductive decomposition of H_2O_2, experiments were performed in the presence of deferoxamine mesylate (DEF), a well-known chelator of iron preventing its further reaction with H_2O_2 [34]. The results showed that the elimination of any trace iron by the addition of DEF (up to 0.01 mM) caused a strong (approximately 60%) decrease in the quantum yield of HPD photobleaching (Table 3). These findings support the view that the photoirradiated HPD can generate $OH^{\bullet}$ via the Fenton reactions.

On the basis of the results obtained, we can propose the following scheme for describing the photobleaching behaviour of HPD in aqueous solution:

$HPD + h\nu \rightarrow {}^1HPD^*$	light absorption
${}^1HPD^* \rightarrow {}^3HPD^*$	intersystem crossing
${}^3HPD^* + O_2 \rightarrow HPD + {}^1O_2$	energy transfer
${}^3HPD^* + O_2 \rightarrow HPD^{+\bullet} + O_2^{-\bullet}$	electron transfer
${}^1O_2 + HPD \rightarrow$ photoproducts	chemical quenching of ${}^1O_2$
$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$	dismutation of superoxide anion radical
$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$	Fenton reactions
$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$	Fenton reactions
$HPD^{+\bullet} + H_2O \rightarrow HPD^{\bullet}\text{–}OH + H^+$	free radical chain reactions
$HPD + HPD^{\bullet} \rightarrow$ photoproducts	free radical chain reactions
$HPD^{\bullet} + O_2 \rightarrow$ photoproducts	free radical chain reactions
$HPD + OH^{\bullet} \rightarrow$ photoproducts	free radical chain reactions
$HPD^{\bullet} + HPD^{\bullet} \rightarrow$ photoproducts	free radical chain reactions
$HPD^{+\bullet} + O_2 \rightarrow$ photoproducts	free radical chain reactions

where ^{1}HPD* and ^{3}HPD* refer to the lowest excited singlet and triplet state of HPD, respectively; HPD$^{\bullet}$ is the neutrally charged radical of HPD; and HPD–OH is the hydroxylated product of the PS.

At least two major reaction pathways were revealed during the study of HPD photodegradation in simple solutions. The first involves $^1O_2$ generation and attack on ground-state HPD, which is confirmed by an increased photobleaching yield in SPB prepared with D_2O and the inhibition effects of NaN_3 on HPD degradation (Table 1). The other pathway appears to involve electron transfer from excited HPD to generate $O_2^{-\bullet}$ and very reactive HPD$^{+\bullet}$. In addition, OH$^{\bullet}$ formed from H_2O_2 through the Fenton reactions may also cause HPD degradation. Thus, our data suggest that HPD can undergo degradation both via type I and type II processes.

Effects of ionic strength, pH, and temperature on HPD photobleaching

Ionic strength and pH

It has been demonstrated that components of HPD form monomers and various aggregates in aqueous solution [26]. A change in the ionic strength of HPD solutions may presumably cause a shift in the equilibrium of its monomeric and aggregated forms. This may affect the light resistance of the antitumour drug, since disaggregation of HPD molecules strongly enhances the quantum yield of $^1O_2$ formation [21]. Our studies indicated that decreasing the SPB (at pH 7.4) concentration from 100 to 0.2 mM had little effect on the quantum yield of HPD photobleaching, reducing it only by 5–8%.

The acidity of the solution is an important factor affecting the equilibrium between the different ionic species of all free base porphyrins, as well as the proportion of monomers, dimers, and larger aggregates of each ionic species. Consequently, the photostability of HPD may be altered by a change in the medium acidity. This is interesting since tumour tissue is acidic in many cases due to excessive lactic acid production [35].

It was found that the dependence of HPD photobleaching on pH has a complex character. Indeed, increasing the solution pH from 7.15 to 11.5 as well as decreasing the pH from 7.15 to 5.0 increased the quantum yields of HPD photobleaching by 77% and 65%, respectively (Fig. 2a). The changes of the non-illuminated HPD Soret band in solutions of various pH show that, in a slightly acidic medium, the PS is more aggregated than in neutral or alkaline solution (Fig. 2b). The growth in the Soret band absorbance as well as its slight shift to the red (from 367 nm at pH 5.0 to 379 nm at pH 11.5) with an increase in pH indicates disaggregation. In the pH region 7.15–11.5 there is a direct relationship between the disaggregation of HPD and photobleaching. However, the steep increase in the quantum yield of HPD photodegradation, particularly around pH 5.5, cannot be explained in this manner. The decreased photostability of HPD in acidic solutions (Fig. 2a) may be induced by a shift in the equilibrium of the ionic species of porphyrins. Seven ionic species caused by the protonation or deprotonation of nitrogen atoms are possible for all free base porphyrins, but

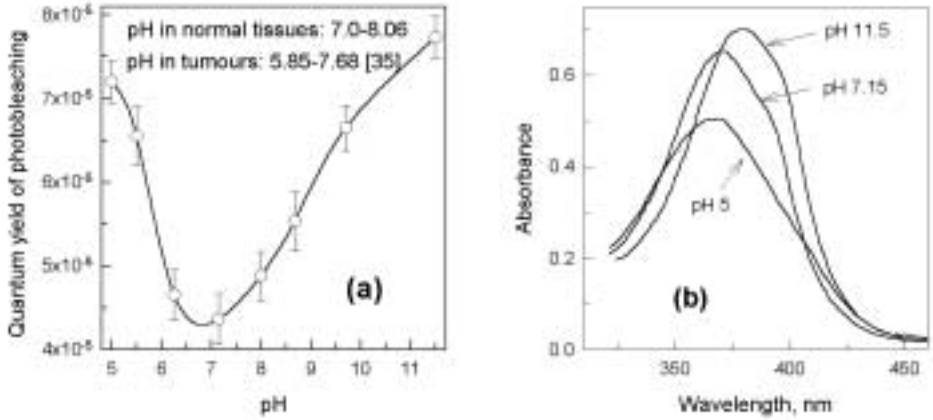

Fig. 2. Quantum yields of HPD (16.7 μM) photobleaching (a) and absorption spectra of the non-illuminated HPD (b) in aqueous solutions (20 °C) at different pH values. The experiments were performed in the buffer prepared by mixing 40 mM solutions of acetic, boric, and phosphoric acids (its pH was regulated by addition of 0.2 M NaOH). Bars are standard errors.

only four (dication and monocation, neutral and dianion) have been observed spectroscopically [26]. HPD, as well as other dicarboxylic porphyrins, can also release one or more protons from its side acid chains. The pH regulates, as known, the predominance of each ionic species and the conversions between them [35]. On the basis of the results obtained we assume that positively charged forms of HPD are less photostable than the neutral or anionic species. This suggestion is in good agreement with the data of other researchers. Móger et al. [1] revealed that the dicationic form of HP dihydrochloride bleaches faster than the neutral species in organic solvent mixtures; studies with a phenolic antioxidant suggest that the photodegradation of the dication is mediated by free radicals.

Additionally we evaluated the effect of pH on the photosensitizing capacity of HPD using Trp as a substrate. It was found that within the whole range of the pH studied (from 5.0 to 11.5) the photosensitizing activity of HPD is closely related to its aggregation state. In fact, decreasing the pH from 7.15 to 5.0 decreased the Soret peak absorbance (Fig. 2b), due to an increased aggregation of the porphyrin, and the quantum yields of HPD-photosensitized oxidation of Trp also decreased (Fig. 3). On the contrary, the disruption of HPD aggregates, which was registered upon raising the pH value from 7.15 to 11.5, increased the yield of Trp photooxidation almost 5-fold (Fig. 3). Thus, the aggregated HPD is a poor PS.

An analysis of the obtained results suggests that the small difference existing between the cancerous and surrounding tissues in the pH region 6–8 cannot alter the photobleaching behaviour of HPD (as can be seen in Fig. 2a), but may significantly decrease its photosensitizing efficiency (as shown in Fig. 3).

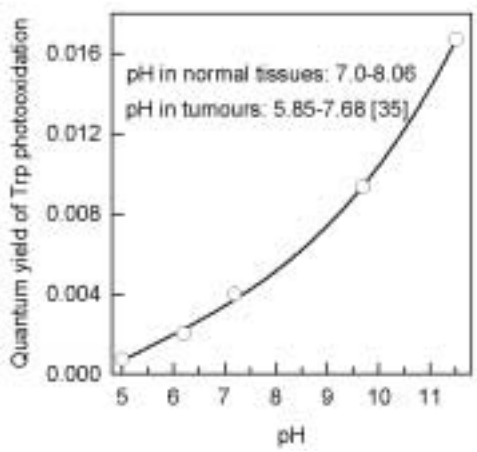

Fig. 3. Quantum yields of HPD (16.7 μM) photosensitized oxidation of 0.2 mM tryptophan in aqueous solutions of various acidity (at 20 °C). The buffer was the same as in Fig. 2.

Temperature

It has been shown that PDT at high light power densities can induce an essential (about 7°C) increase in the temperature of tumour tissues [36]. This heating of a neoplasm might promote the photobleaching of HPD. However, in the literature we did not find any information about an influence of temperature on the photobleaching behaviour of the drug. In the work we estimated, therefore, the effect of temperature on the photostability of HPD in simple solution.

We found that the photodegradation of HPD in aqueous solution was only slightly dependent on temperature. In fact, raising the temperature from 10 to 43 °C caused only a moderate (approximately 2-fold) increase in the quantum yield of HPD photobleaching (Table 4). Moreover, in the temperature region of special interest (from 36 to 43 °C) the increase in the yield of HPD photobleaching was negligible (about 10%). According to our calculations, the activation energy of HPD photodegradation in aqueous solutions (at pH 7.4) was low (about 3.8 kcal/mole). Studies on the mechanism suggest that the stimulant effect of temperature on HPD photobleaching could be partly explained by disruption of its

Table 4. Influence of temperature on the quantum yield of HPD photobleaching and HPD-sensitized photooxidation of 0.2 mM tryptophan (the reaction mixtures were 16.7 μM in HPD, 100 mM in SPB of pH 7.4 and air-equilibrated)

Temperature, °C	Quantum yield of HPD photobleaching	Quantum yield of Trp photooxidation
10	2.77×10^{-5}	3.30×10^{-3}
20	3.61×10^{-5}	4.89×10^{-3}
30	4.45×10^{-5}	6.57×10^{-3}
37	5.01×10^{-5}	8.58×10^{-3}
43	5.58×10^{-5}	10.31×10^{-3}

dimers and larger aggregates, since increasing the temperature from 10 to 43 °C was associated with a notable red shift in the position of the non-illuminated HPD Soret peak (data not shown). Perhaps the thermal disaggregation of HPD molecules enhanced the formation of porphyrin destroying $^1O_2$. Indeed, a rise in the temperature of the reaction mixture increased (as shown in Table 4) the quantum yield of HPD photosensitized oxidation of Trp (a well-known chemical quencher of $^1O_2$ [23]). The activation energy of the Trp photooxidation was determined as 6.2 kcal/mole.

Effects of miscellaneous agents on HPD photobleaching

HPD and PII are lipophilic and are concentrated mainly in cellular membranes [37]. To mimic the photobleaching behaviour of HPD in biological systems, we investigated its degradation in the presence of various detergents (like cell membranes the micelles of surfactants have low dielectric constants). The non-ionic detergent, Triton X-100 (TX-100), reduced the initial quantum yield of HPD photobleaching in 0.1 M SPB (pH 7.4) 26% at 0.25 mM. The cationic detergent, cetyltrimethylammonium bromide (CTAB), decreased the yield 63% at 1.0 mM, whereas sodium dodecyl sulphate (SDS), an anionic detergent, reduced the yield 50% at 8.27 mM (Table 5). Thus, all studied surfactants, at a critical micelle concentration, strongly enhanced the light resistance of HPD. Spectral measurements showed that HPD penetrates into micelles of these detergents; a typical red shift of the Soret band (from 369 to 402 nm) upon increasing the concentrations of TX-100, CTAB, and SDS was observed (Fig. 4). Thus, we cannot explain the increased photodestruction of HPD in tumour cells [15, 16] by its inclusion in domains (membranes) having low dielectric constants, although such a process may enhance (due to significant monomerization of the porphyrin) the formation of $^1O_2$. In fact, it was found that CTAB, at a concentration of 1.0 mM, caused a powerful (more than 5-fold) increase in the quantum yield of HPD-sensitized photooxidation of 0.2 mM Trp (data not shown).

Table 5. Quantum yields of HPD (16.7 μM) photobleaching in the presence of various detergents relative to the yield in air-equilibrated 100 mM SPB (pH 7.4) at 20 °C (the initial quantum yield is given in Table 2)

TX-100, mM	Relative quantum yield of photobleaching	CTAB, mM	Relative quantum yield of photobleaching	SDS, mM	Relative quantum yield of photobleaching
Buffer	1.00	Buffer	1.00	Buffer	1.00
0.25*****	0.74	0.10	0.56	5.0	0.51
1.0	0.72	1.0*****	0.37	8.27*****	0.50
5.0	0.67	5.0	0.34	20.0	0.47

***** critical micelle concentration.

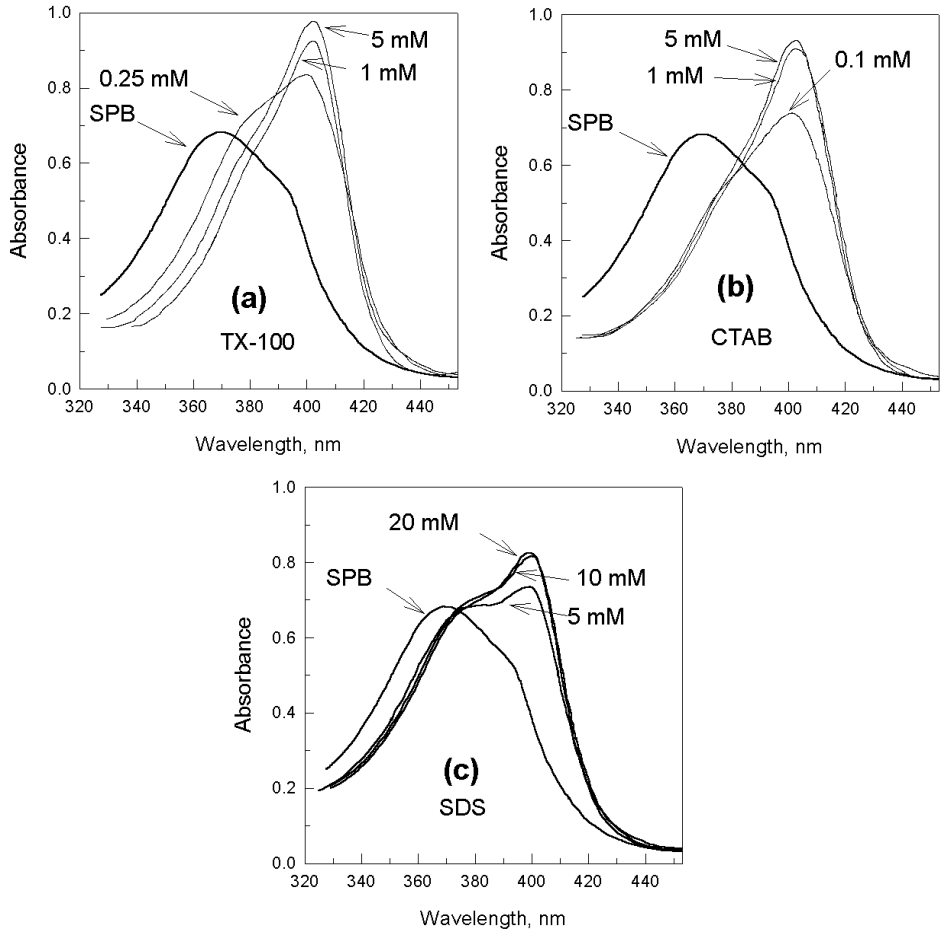

Fig. 4. Absorption spectra of the non-illuminated HPD (16.7 μM) in 0.1 M SPB of pH 7.4 (20 °C) at different concentrations of: (a) Triton X-100 (TX-100), (b) cetyltrimethylammonium bromide (CTAB), and (c) sodium dodecyl sulphate (SDS) (sample light path, 0.5 cm).

Effects of photooxidizable substrates on HPD photodegradation

Sensitizers in body fluids and tumours can be closely associated with a wide variety of biomolecules that are susceptible to photosensitized oxidation, including amino acids, proteins, unsaturated lipids, nucleic acids, etc. [38]. These might interact with porphyrins in various ways to alter the yields and mechanisms of photobleaching. Indeed, one of our earlier studies [16] showed that HPD internalized into Ehrlich carcinoma cells photobleaches faster than in aqueous buffer. We also found that HPD is photodegraded faster in the presence of bovine serum albumin than in phosphate-buffered saline [39]. It has been shown by others [40] that some porphyrins photobleach more rapidly when incorporated into erythrocyte ghosts (which contain photooxidizable lipids and

proteins) or in microemulsions containing photooxidizable amino acids than in pure solvent. Roberts et al. [41] showed that certain thiols, including cysteine, glutathione (reduced), and aminoethylthiol, increase the photobleaching rate of tetra(4-sulphonatophenyl)-porphine in solution. In the present work, the effects of several photooxidizable organic compounds on the bleaching of HPD were, therefore, examined.

Concentration effects of the photooxidizable amino acids (histidine, methionine, and Trp) on the quantum yield of HPD photobleaching are shown in Table 6. The concentrations of Trp that could be studied were limited because of the formation of photoproducts that interfered with spectroscopic measurements of HPD during photobleaching. At low concentrations (from 0.01 up to 0.1 mM), all three amino acids slightly inhibited the photobleaching yields of HPD. High concentrations of methionine and histidine (from 5 to 10 mM) decreased the quantum yield of HPD photodegradation essentially. However, intermediate concentrations of methionine and histidine had opposite effects. In fact, methionine (1.0 mM) decreased (by 35%), while histidine (at the same concentration) increased the photobleaching yield of HPD by 43%. It is important to note that arginine (a non-photooxidizable amino acid) had no effect on the light resistance of HPD in aqueous solution even at 10 mM (data not shown).

HPD can photooxidize cysteine by type I and/or type II processes, depending on reaction conditions [42]. As shown in Table 6, cysteine inhibited the photobleaching of HPD at all concentrations studied (from 0.01 up to 10 mM). Reduced glutathione and dithiothreitol, at concentrations from 0.01 up to 1.0 mM, slightly increased the quantum yield of HPD photobleaching. However, high concentrations of the thiols (>1.0 mM) decreased the quantum yield of HPD photodegradation. The disulphides, oxidized glutathione, and cystine had no effect on photostability of HPD (data not shown) even at the highest concentration used (10 mM). Krieg & Whitten [40] suggested that the increased rates of porphyrin photobleaching in the presence of the photooxidizable substrates (diethylsulphide, histidine, cysteine, methionine, and Trp) could result

Table 6. Relative quantum yields of HPD photobleaching as a function of the concentrations of various photooxidizable substrates. The reaction mixtures were 16.7 μM in HPD, 100 mM in SPB of pH 7.4, and 0.28 mM in oxygen (air-equilibrated). The temperature was 20 °C. The control value for HPD photobleaching is given in Table 2

Substrate	Substrate concentration, mM				
	0.01	0.1	1.0	5.0	10
Histidine	0.87	0.79	1.43	0.38	0.07
Methionine	0.94	0.9	0.65	0.47	0.25
Tryptophan	0.96	0.59	–	–	–
Cysteine	0.92	0.91	0.9	0.34	0.27
Glutathione	1.12	1.06	1.03	–	0.79
Dithiothreitol	1.0	1.10	1.03	0.83	0.43
NADH	0.55	0.03	–	–	–

from an attack on the porphyrin macrocycle by reactive $^1O_2$ photooxidation products of the substrates. In the present work, the reasons for the markedly different effects of some of these compounds on the photobleaching yields of HPD are not clear.

NADH, a biological component and an electron donating agent, had a large effect on the photobleaching yield of HPD, decreasing it over 30-fold at 0.1 mM (Table 6). At this concentration, NAD^+ (an oxidized form of NADH) had no effect on the rate of HPD self-photosensitized oxidation. Perhaps the effect of NADH on photodegradation of HPD is mediated by inhibiting the very reactive $HPD^{+\bullet}$ formation.

Furthermore, we investigated the effects of cholesterol and lecithin on the photobleaching behaviour of HPD. These lipids are the main components of cellular membranes and can be oxidized under photoexcitation of the PS in tumour cells [43]. In ethanol, the quantum yield of HPD photobleaching was practically unchanged by the presence of cholesterol (5 mM), while the addition of lecithin (up to 3 mg/mL) increased the yield almost 2-fold (data not shown). These findings suggest that under PDT the formed phospholipid hydroperoxides may catalyze the photodegradation of HPD in tumour tissue.

Effects of electron-accepting compounds on HPD photobleaching

Triplet state porphyrins tend to be better electron donors and acceptors than the ground state molecules [24, 26]. Cells and tissues contain both electron acceptors and donors. These compounds might alter the photobleaching behaviour of HPD. In the work, we already demonstrated that certain electron donors are able to inhibit the self-sensitized photooxidation of HPD. Indeed, the quantum yields of HPD photobleaching were substantially reduced by the presence of cysteine and NADH (Table 6). In further studies, we evaluated the effects of selected model electron acceptors on the photobleaching yields of HPD.

Quinones can abstract an electron from triplet porphyrins, giving a radical cation of the porphyrin and a radical anion of the quinone [44]. 1,4-Benzoquinone decreased the quantum yield of HPD photobleaching at all concentrations studied (from 0.001 up to 0.25 mM) (Table 7).

Methylene blue (MB), an organic compound bearing a diffuse positive charge, can undergo one electron reduction under photoexcitation of some porphyrins with the formation of a colourless material (leucoMB) [45]. In the presence of molecular oxygen the formed leucoMB is rapidly oxidized producing $O_2^{-\bullet}$ and H_2O_2 [46]. MB decreased the quantum yield of HPD photobleaching more than 5-fold at 0.01 mM (Table 7). At all concentrations studied, MB caused a decrease in the absorbance and fluorescence of HPD solutions, shifting the Soret peak of the PS to longer wavelengths (Fig. 5). This suggests that a strong decrease in the photobleaching efficiency of HPD may result from electrostatic binding and/or aggregation of the positively charged MB with the negatively charged molecules of HPD.

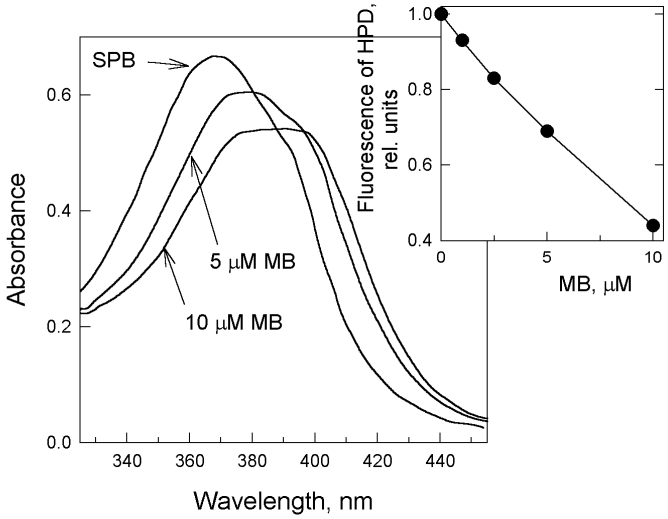

Fig. 5. Absorption spectra of the non-illuminated HPD (16.7 μM) in 0.1 M SPB of pH 7.4 (20 °C) at different concentrations of methylene blue (MB) (sample light path, 0.5 cm). Inset: fluorescence intensities of HPD solutions at 617 nm (λ_{ex} = 400 nm) under the same conditions.

Flavin mononucleotide (FMN), which may be photoreduced under illumination of some tetrapyrrolic compounds to the corresponding semiquinone radical [45], increased slightly the quantum yield of HPD photobleaching (Table 7).

Metronidazole, an electrophilic nitroimidazole "radiosensitizer", inhibits $^1O_2$ formation by illuminated porphyrins reacting with their triplets at a diffusion-controlled rate to give the porphyrin radical cation and the metronidazole radical anion [47]. It must be emphasized that the life-time of the radical cation is much longer than that of $^1O_2$. Metronidazole had a large effect on the photobleaching yield of HPD, increasing it over 30-fold at 0.1 mM (Table 7).

Table 7. Relative quantum yields of HPD photobleaching as a function of the concentrations of various electron acceptors. Reaction conditions, except for substrate, were the same as described in Table 6 (the control quantum yield is given in Table 2)

Electron-accepting compound	Concentrations of electron acceptors*, mM				
	0.001	0.005	0.01	0.1	0.25
1,4-Benzoquinone	0.87	–	0.75	0.63	0.39
Methylene blue	0.91	0.48	0.18	–	–
Flavin mononucleotide	0.99	–	1.4	–	–
Metronidazole	1.4	–	9.1	34.5	–

***** low concentrations of the compounds were used to avoid interference with measurements of the porphyrin absorption during photobleaching.

Thus, at certain concentrations, the quantum yield of HPD photobleaching was slightly enhanced by FMN and significantly increased by metronidazole. However, benzoquinone and MB did not increase the yield over the concentration range examined. The mechanisms of the increased yield of HPD photobleaching in the presence of metronidazole are unclear and need further studies. Nevertheless, it could be assumed that metronidazole enhances the production of very reactive $HPD^{+\bullet}$. On the other hand, the formed metronidazole radical anion might react with HPD, converting it to colourless products (the radical anion is known to react with biomolecules such as Trp [47]). In addition, the metronidazole anion radical reacts with ground state oxygen with the formation of $O_2^{-\bullet}$ [47]. This event, in turn, may cause the generation of H_2O_2 and $OH^{\bullet}$. The latter, as found by using corresponding traps (Table 3), is involved in the photobleaching of HPD in aqueous solution. Because tissues contain a large number of electron acceptors, photobleaching of HPD in vivo may be affected by reactions of this type.

Effects of biological antioxidants on HPD photobleaching

Ascorbic acid (ASA) and α-tocopherol (α-TOC) are the most important biological antioxidants. ASA, a compound highly soluble in water, reacts with free radicals that arise in the aqueous compartments of tissues, forming innocuous ascorbate semiquinone. α-TOC, a highly lipophilic molecule, is the main antioxidant in biological membranes. It reacts with free radicals to form very stable tocopherol semiquinone. At the same time, it was demonstrated that ASA generates H_2O_2 and $OH^{\bullet}$ upon illumination of HPD in aqueous solutions [48], while α-TOC acts as an effective scavenger of $^1O_2$ by physical quenching and chemical reactions [49]. Thus, it is of interest to study the effects of ASA and α-TOC on the photobleaching behaviour of HPD.

As shown in Table 8, the quantum yields of HPD photobleaching were considerably reduced only at high concentrations (>1 mM) of ASA and α-TOC, while low concentrations (from 0.001 up to 0.1 mM) of these biological antioxidants had little or unexpected effects on the photobleaching yields. To our surprise, α-TOC, at a concentration of 0.1 mM, caused a substantial (approximately 70%) increase in the quantum yield of HPD photobleaching. In this work, we found that under photoexcitation of HPD, α-TOC undergoes a rapid oxidation with the formation of products absorbing light in the spectral region of 317–360 nm with a clearly expressed maximum at 317 nm (data not shown). Further studies showed that the photoproducts of α-TOC may act as sensitizers. In fact, when the reaction mixture was irradiated with light at wavelengths >455 nm, the presence of α-TOC (0.1 mM) mediated a 1.5 decrease in the rate of HPD photodestruction.

Thus, in the present work we showed that both ASA and α-TOC inhibit the photobleaching of HPD in solution. However, at low concentrations (from 0.01 to 0.1 mM) of these biological antioxidants, which occur in vivo [48, 50], their inhibitory effects were relatively small. Moreover, at certain conditions α-TOC can promote the photobleaching of HPD by the formation of products that have photosensitizing activity.

Table 8. Relative quantum yields of HPD (16.7 μM) photobleaching as a function of the concentrations of ascorbic acid (in 100 mM SPB of pH 7.4) and α-tocopherol (in DMFA) at 20 °C. The control values for HPD photobleaching are given in Table 2

Antioxidant	Antioxidant concentrations, mM					
	0.001	0.01	0.1	1.0	5.0	10
Ascorbic acid	0.96	0.91	0.86	0.44	0.18	0.12
α-Tocopherol	0.98	0.96	1.68	0.74	0.32	0.23

CONCLUSIONS

On the basis of the results obtained we made a few general conclusions:

1. Various investigators [20, for instance] suggest that the photobleaching of HPD in simple solution is mediated by $^1O_2$ reaction with the ground state of the PS. However, our studies showed that besides $^1O_2$, free radical reactions are involved in the photodegradation of HPD in aqueous solution. Indeed, the quantum yield of HPD photobleaching was decreased in the presence of $OH^{\bullet}$ scavengers, such as mannitol, sodium benzoate, ethanol, and DEF. Furthermore, our data suggest that the photodegradation of HPD could be associated with the formation of very reactive cation radicals of the sensitizer.

2. The small difference existing between cancerous and surrounding tissues in the pH region 6–8 cannot alter the efficiency of HPD photobleaching (as can be seen in Fig. 2a). Furthermore, our findings suggest that positively charged forms of HPD are less photostable than the neutral or anionic species.

3. The photostability of HPD in solution is only slightly dependent on temperature; in aqueous buffer (pH 7.4) raising the temperature from 10 to 43 °C caused only a moderate (about 2-fold) increase in the quantum yield of HPD photobleaching. Studies on the mechanism suggest that the enhancing effect of temperature on HPD photobleaching could be partly explained by the disruption of its dimers and larger aggregates. Perhaps the thermal disaggregation of HPD molecules enhanced the production of porphyrin destroying $^1O_2$.

4. The polarity of the reaction mixture is a major determinant of the efficiency of HPD photodestruction. For instance, a decrease in the medium polarity (upon addition of organic solvents to aqueous buffer) strongly enhanced the light resistance of HPD (Fig. 1). Thus, the increased photodegradation of HPD in tumour cells [15, 16] (as compared to photobleaching in aqueous buffer) cannot be explained by its inclusion in domains (membranes) having low dielectric constants.

5. Some photooxidizable substrates and model electron acceptors can increase the photobleaching efficiency of HPD substantially. In fact, at certain concentrations, the quantum yield of HPD photobleaching was enhanced by the presence of histidine, reduced glutathione, dithiothreitol, and lecithin. Electron accepting compounds, such as metronidazole and FMN, also increased the photobleaching yield. In contrast, NADH and cysteine, which are electron

donors, inhibited the rate of HPD photodestruction. Since cells and tissues contain a wide variety of photooxidizable biomolecules, electron acceptors and donors, the mechanism of the photobleaching of HPD during the PDT of tumours is complex, and would be expected to depend on the chemical composition of the sites where the sensitizer localizes in the cells and tissues.

6. Biological antioxidants, such as vitamin C and α-TOC, can potentially inhibit the photobleaching of HPD in cells and tissues. However, it was found that α-TOC is oxidized by HPD and light with the formation of products that at certain conditions can photosensitize the porphyrin bleaching.

7. The aggregated HPD is a poor PS. Indeed, under increasing the temperature (from 10 to 43 °C) and the pH value (from 5.0 to 11.5) or after addition of various surfactants, the disruption of HPD aggregates strongly enhanced the photosensitizing capacity of the porphyrin towards Trp (a well-known target for oxidation by PDT).

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Hematoporfüriini derivaadi fotodegradatsiooni mehhanismi kineetilised uuringud

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Hematoporfüriini derivaat (HPD), kasvajate fotodünaamilises teraapias kasutatav fotosensibilisaator, laguneb kiiritamise käigus. Porfüriinsete sensibilisaatorite fotolagunemise mehhanism ei ole veel selge, kuigi sellel nähtusel võib olla nii kahjulik kui ka kasulik mõju. Töös hinnati reaktsioonitingimuste, fotolagunemisele alluvate biomolekulide, elektroniaktseptorite ja teiste agentide toimet HPD lahuste fotodegradatsiooni kvantsaagisele (QY) ja kineetikale. Fosfaatpuhvris (pH 7,4) määrati õhuhapniku manulusel HPD fotolagunemise QY-ks $3{,}6 \times 10^{-5}$. QY vähenes oluliselt madala dielektrilise läbitavusega orgaanilistes solventides ja pindaktiivsete ainete manulusel. Ioontugevuse, pH ja temperatuuri toime HPD fotolagunemisele oli suhteliselt nõrk. Temperatuuri tõus 10 °C-lt 43 °C-ni tingis ainult kahekordse HPD QY suurenemise. HPD fotodegradatsiooniks oli vajalik hapniku juuresolek. D_2O keskkond suurendas HPD QY-t üksnes 55%. Naatriumasiid (efektiivne singletse hapniku kustutaja) mõjutas isegi kontsentratsioonil 50 mM HPD fotolagunemise QY-t vähe. Saadud andmetest tulenes, et peale singletse hapniku toimub HPD fotodegradatsioon vesilahustes vabaradikaalsete reaktsioonide kaudu. Hüdroksüülradikaalide aktseptorite (mannitooli, naatriumbensoaadi, etanooli ja deferoksamiini) lisamine vähendas HPD fotolagunemise QY-t. HPD fotodegradatsioon on nähtavasti seotud väga reaktiivsete porfüriini katioonradikaalide tekkega. Kergesti fotolagunevad substraadid ja mõned elektroniaktseptorid suurendasid oluliselt HPD fotolagunemist. Mõnedel kontsentratsioonidel tõstsid histidiin, taandatud glutatioon, ditiotreitool ja letsitiin HPD fotolagunemise QY-t. Sama tegid elektroniaktseptorid metronidasool ja flaviinmononukleotiid. Vastupidiselt toimisid aga elektronidoonorid NADH ja tsüsteiin, inhibeerides HPD fotolagunemist. Bioloogilised antioksüdandid askorbiinhape ja α-tokoferool tõstsid suurtel kontsentratsioonidel (>1 mM) HPD lahuste fotostabiilsust. Saadud tulemused viitavad, et HPD fotodegradatsiooni mehhanism(id) rakkudes ja koes on keerukad.