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PHOTOOXIDATION OF HEMATOPORPHYRIN DERIVATIVE–BOVINE SERUM ALBUMIN COMPLEX

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Abstract. At the illumination (λ_{max} = 405 ± 20 nm; fluence rate 27.5 mW/cm^2) of hematoporphyrin derivative (HpD) in aqueous solution (pH 7.4) several photoprocesses take place: photosensitized destruction of the porphyrin macrocycle, resulting in a loss of absorbance and fluorescence, and photochemical modifications leaving the porphyrin ring intact. In fact, the formation of photoproducts that have an absorption maximum around 640 nm (photoproduct-644) was observed. Perhaps photoproduct-644 is a chlorin-type molecule formed predominantly from the aggregates of porphyrins when photooxidation and photoreduction are in competition. The efficiency of photoproduct-644 formation strongly increased in the presence of NADH and ascorbic acid (the proton donors). On the contrary, its formation was suppressed when bovine serum albumin (BSA) was added to HpD aqueous solution. In 0.05% BSA about 2-fold enhancement in the initial rate of HpD photobleaching was observed when a fall of the porphyrin absorption in the Soret peak was measured. We assume that the binding of HpD to BSA, leading to monomerization of the aggregated sensitizer molecules, was accompanied with an increase in the quantum yield of the HpD triplet state and, consequently, with the formation of destroying porphyrins singlet oxygen. We found that BSA is very susceptible to HpD photosensitized modification. At light doses that are usually used in the phototherapy of tumours substantial photooxidation of tryptophan and tyrosine residues was detected. Surprisingly, after the illumination of the HpD–BSA system the affinity of BSA to HpD was enhanced. It was expressed as a considerable decrease in the distribution coefficients of HpD between 1-octanol and BSA solution with the illumination time.

Key words: photodynamic therapy, tumour, hematoporphyrin derivative, photobleaching, binding, serum albumin.

Abbreviations: PDT = photodynamic therapy; HP = hematoporphyrin IX; HpD = hematoporphyrin derivative; PII = photofrin-II; PP = protoporphyrin; PS = photosensitizer; $^1O_2$ = singlet oxygen; HSA = human serum albumin; BSA = bovine serum albumin; Trp = L-tryptophan; AA = L-ascorbic acid; k_d = coefficient of the distribution of HpD between 1-octanol and BSA solution.

INTRODUCTION

Hematoporphyrin derivative (HpD) and the purified product photofrin II (PII) are the most widely used photosensitizers (PS) for photodynamic therapy of cancer (PDT). HpD is a mixture of porphyrins prepared from hematoporphyrin IX (HP) by acetylation and hydrolysis under basic conditions. Usually this mixture consists of approximately 20% HP, 20–30% hydroxyethyl vinyl deuteroporphyrin, 3–5% protoporphyrin (PP). The remaining about 50% appears to be an oligomeric mixture not yet fully defined chemically. PII is a mixture enriched in this latter fraction to the extent of about 80–90% [1]. This fraction (containing covalent dimers, trimers, etc.) is responsible for the in vivo photodynamic activity of HpD and PII [1, 2].

The clinical treatment regimen usually consists of the intravenous administration of HpD followed by an equilibration period of 24–72 h, during which the "biologically active" porphyrin components of this PS are retained in tumour loci at greater concentrations than in some, but not all, normal tissues [3]. Subsequently the malignancies are exposed to visible radiation, usually 630 nm laser light. Photoexcitation of the tumour-localizing components of HpD leads to the production of singlet oxygen ($^1O_2$), which is reported to be the main agent responsible for induction of necrosis and regression of malignancies [2].

Research into sensitizer–protein interactions is urgently needed to improve clinical PDT. It has been shown by other authors [2, 3] that the concentration of HpD or PII in tumour cells, the distribution of PS in neoplastic tissue and in organism, and the mechanisms of PDT-mediated tumour eradication can depend on the binding of PS with several extra- and intracellular proteins.

It has been shown that the uptake of HP in tumour cells in vitro and PII in vivo is enhanced by exposure to light [4, 5]. Therefore, it was supposed that the tumour-to-normal-tissue concentration ratio of PII can be improved by means of exposing the tumour area to a small dose of light at a time when the concentration of PII in the blood is high [5]. However, the effect of irradiation on the tumour uptake of PS is not clearly understood. It is possible that the light-induced increase in HpD uptake by the tumour cells may be the result of covalent binding of HpD components with several cellular molecules, probably with protein. However, another mechanism of the light-mediated increase in the HpD level in the tumour loci may occur. Albumin is the most important porphyrin-binding protein in serum. Under controlled experimental conditions, human serum albumin (HSA) forms stable 1:1 complexes with a number of porphyrins [6]. However, experimentally determined uptake of PII by NHIK 3025 cells from solutions containing different amounts of HSA showed that the amount of PII bound to the cells is proportional to the free PII [7]. Proteins are subjected to photosensitized modification by $^1O_2$ [8]. Perhaps the release of HpD components from their complex with the protein, caused by the photochemical damage of the porphyrin-binding sites in the serum albumin, led to a growth of PS contents in the cancerous tissue. However, these speculations about

mechanisms of the light mediated growth of the HpD concentration in tumour need experimental checking.

The study of the HpD-photosensitized degradation of not only serum but also of some cellular proteins is of special interest because the process of PDT-induced tumour necrosis may be mediated by preferential destruction of such biomolecules [2, 8]. In this study serum albumin was used (as a model) for the examination of HpD-photosensitized destruction of protein. Serum albumin represents an ideal object since it possesses a single L-tryptophan (Trp) residue, whose photooxidative modification can be readily followed by fluorescence spectroscopy [9].

Light degrades many of the PSs that have been used in biomedical studies, usually with the formation of products that do not absorb strongly in the visible range and which do not act as a PS. This process, called photobleaching, can occur with dyes in solution and in body fluids, cells, and tissues. In principle, the photobleaching of the sensitizers used in the PDT of tumours can be a disadvantage or an advantage. Indeed, if the sensitizer is bleached too rapidly as a result of illumination during therapy, tumour destruction may be incomplete. On the other hand, patients undergoing HpD–PDT must remain indoors and out of direct sunlight for about a month to avoid sunburn-like effects of their skin due to its photosensitivity [10]. A possible method of quick elimination of skin photosensitivity is the use of photobleaching to remove the PII from the skin [10]. Until recently, it was assumed that porphyrins are photochemically stable during the treatment. Moan et al. [11, 12] were the first to demonstrate the possible photooxidation of HpD in cells treated in culture. Similar photobleaching of PII has been observed in patients undergoing PDT [13]. One may suppose that the rate of HpD photodestruction in tumour cells is higher than in simple aqueous solutions. It was reported that in cells a large fraction of the porphyrins are in close contact with proteins [12]. Thus, it is likely that binding to cellular proteins enhances porphyrin photobleaching. Therefore, it is of interest to examine the photobleaching behaviour of sensitizers proposed for use in PDT, especially in the presence of a porphyrin-binding protein.

The aim of the present work was to investigate (1) the kinetics and mechanism of HpD photodestruction in the presence of serum albumin; (2) the patterns in HpD-photosensitized modification of serum albumin; and (3) the effect of exposure to light on the stability of HpD-albumin complex.

MATERIALS AND METHODS

Chemicals

HpD was synthesized from hematoporphyrin IX dihydrochloride (Aldrich) according to the original method of Lipson et al. [14] modified by Kessel et al. [15]. Bovine serum albumin (BSA) fraction V, essentially fatty acid-free, and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Irradiation procedure

In all the experiments a 1 kW xenon arc-lamp, which has a continuous character of the light emission from 200 nm up to infrared domain of the solar spectrum, equipped with an interference filter, which transmits 48% of light at 405 nm (with half bandwidth 20 nm), and a focusing optical system served as the radiation source. The flux of light was focused as a spot (D = 1.8 cm) and directed on the front face of a quartz cuvette containing HpD solutions. The intensity of the emitted light at 405 nm was always 70 mW with a power density 27.5 mW/cm^2, as measured with an IMO-1 radiometer (Russia).

Study of HpD phototransformations without or in the presence of bovine serum albumin, NADH, histidine, tryptophan, ascorbic acid, and D,L-buthionine-[S,R]-sulphoximine

In a typical experiment, 8 ml of a 10 μg/ml HpD aqueous solution without or jointly with other agents (supplemented with a 10 mM Tris-HCl buffer, pH 7.4) was placed in a quartz cuvette of 2 cm optical path. Then the samples were irradiated under magnetic stirring at 20 °C. The fall of the porphyrin absorption in the Soret peak (around 400 nm) and of the fluorescence intensity in the 614 nm band were measured to determine the rate of HpD photodestruction. These measurements were performed with a spectrophotometer SPECORD M-40 and a spectrofluorimeter Hitachi 650-40. The kinetics experiments were repeated three times. The magnitude of the standard error was less than 5% for the kinetics measurements.

Photodestruction of L-tryptophan and bovine serum albumin

The absorbance (differential spectrum) of Trp and BSA solutions at 280 nm was measured to determine their reactivity under HpD (initial concentration was 10 μg/ml) photoexcitation. The efficiency of HpD photosensitized oxidation of Trp residues in BSA was estimated by means of measurements of the fluorescence emission intensity of this protein solution at 342 nm (λ_{ex} = 280 nm). The irradiation was carried out in the quartz cuvette (optical path, 2 cm; volume, 8 ml) under continuous magnetic stirring at 20 °C.

Assay for the stability of the HpD–BSA complex under photooxidation

The coefficient of the distribution of HpD between 1-octanol and BSA solution (k_d) was evaluated to determine the stability of the HpD–BSA complex during its illumination. In particular, 8 ml of a 0.2% BSA aqueous solution (supplemented with 10 mM sodium phosphate buffer, pH 7.4) together with 10 μg/ml HpD, was placed in a quartz cuvette with a 2 cm optical path. The solution was air-equilibrated and thermostated at 20 °C by water circulation through the cell holder. After illumination (under magnetic stirring) the fractions of 3 ml were withdrawn and added to 3 ml of 1-octanol for the extraction (by shaking during 5 min) of HpD. Further the samples were centrifuged at 3000 × g for 10 min at room temperature to separate the 1-octanol phase from the BSA solution. The concentrations of HpD in the two phases were determined

spectrophotometrically by measuring the absorbance at the Soret band and then the partition coefficient was calculated. The experiments for k_d determination were repeated three times.

RESULTS AND DISCUSSION

Photodestruction of HpD in the absence of serum albumin

Figure 1 indicates that illuminations ($\lambda_{max} = 405 \pm 20$ nm) of HpD in aqueous solution led to the photodegradation of its molecules, as proved by the decrease of the absorption maximum in the Soret peak (a consequence of the splitting of the tetrapyrrolic ring). The fluorescence emission intensity of HpD (at 614 nm) also decreased during bleaching (Fig. 1*b*). The time course of HpD photobleaching was essentially first order. Moreover, some unexpected results were obtained during the study of the kinetics of self-sensitized photooxidation of HpD. Thus, the initial rate of HpD photobleaching evaluated by measuring the porphyrin absorption in the Soret band was approximately 3-fold smaller than that obtained by measurements in the fluorescence emission of HpD aqueous solutions at 614 nm (Fig. 2). Such difference in the rates of HpD photobleaching is difficult to explain. Nevertheless, it is well known that the HpD components, including covalently (ether/ester) bound dimers, exhibit a strong tendency to self-associate in aqueous solution because of their planar structure and hydrophobic character that favour electrostatic interactions and thus a stacking effect [16, 17]. Moreover, the fluorescence and triplet state yields of porphyrins can be substantially affected by aggregation (a consequence of self-quenching) [18]. Measurements performed on HpD in aqueous solution showed that the fluorescence emission spectrum can be ascribed mainly to the monomeric species, while the absorption spectrum exhibits both monomers and aggregates [19]. Therefore, a more rapid decrease of the fluorescence emission (at 614 nm) in HpD aqueous solution than the porphyrin absorption (in the Soret band) may be the result of preferable (in comparison with aggregates) photodestruction of the monomeric species. In fact, the monomeric porphyrin species excited on illumination can produce $^1O_2$, which induces subsequent photochemical reactions leading to porphyrin oxidation followed by the destruction of the tetrapyrrolic ring [20]. On the contrary, in large aggregates essentially anaerobic conditions may be created, since it is very difficult for oxygen to penetrate into the tightly packed aggregates by diffusion. As a consequence, the photooxidation of HpD by oxygen in aggregates is inhibited. On the other hand, because the intensity of porphyrin fluorescence may partly depend on its aggregate states, we propose that the degree of molecular aggregation in the HpD aqueous solution strongly increased with the illumination time. Perhaps the phototransformation of HpD was associated with the formation of the porphyrin species, which owing to a large tendency for self-aggregation has a lower quantum yield of the light-induced fluorescence (at 614 nm) than the initial HpD components.

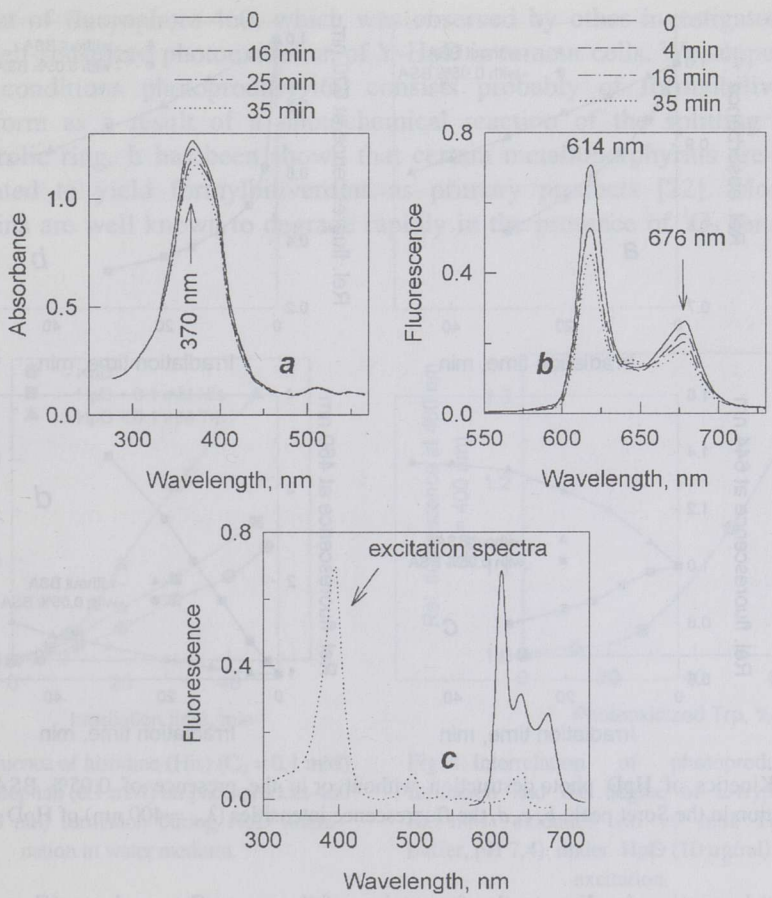

Fig. 1. Absorption (*a*) and fluorescence emission (λ_{ex} = 400 nm) (*b*) spectra of non-illuminated or illuminated HpD in aqueous solution; (*c*) the excitation (λ_{em} = 644 nm) and fluorescence (λ_{ex} = 494 nm) spectra of photoproduct-644.

Figure 1 demonstrates the initial fluorescence spectrum of HpD in water medium at pH 7.4 (with peaks at 614 and 676 nm). After illumination, two new fluorescence peaks appeared. The first fluorescence band is observed at 644 nm, which exhibits a maximum intensity on excitation at 494 nm. On excitation at 394 nm, the second fluorescence peak (is very weak) lay in the 456 nm band. It could be supposed that the appearance of the new fluorescence bands (in the HpD-fluorescence spectrum) is associated with the formation of photoproducts, since the fluorescence emission intensity at 456 nm (photoproduct-460) and at 644 nm (photoproduct-644) had an apparent tendency to increase at prolonged light exposure (Fig. 2). Cao et al. [21] also observed a fluorophore emitting at 460 nm (upon excitation at 365 nm) after irradiation of tumour cells and human cancer tissues in the presence of Y-HpD or PII. They showed that the photo-

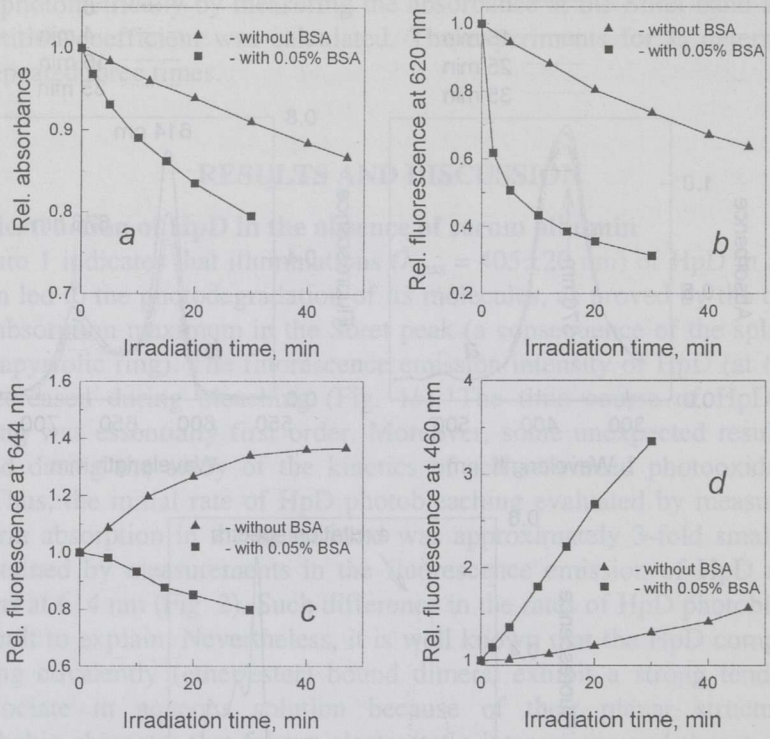

Fig. 2. Kinetics of HpD photo destruction without or in the presence of 0.05% BSA: *a*, HpD absorption in the Soret peak; *b*, *c*, *d*, the fluorescence intensities (λ_{ex} = 400 nm) of HpD solution.

chemical reaction leading to the formation of the same fluorophore (fluorophore-460) is highly specific, since no fluorescence at 460 nm appeared in the absence of histidine, or when Y-HpD was replaced by non-porphyrin PS. These authors proposed that a covalent linkage is formed between the histidine or photooxidized histidine residues of proteins and porphyrin (or photooxidized porphyrins). However, in our experiments, the addition of histidine into the reaction system did not stimulate similar formation of photoproduct-460 (Fig. 3). An acceleration of photoproduct-460 formation was observed when illumination of HpD solutions was carried out in the presence of Trp (Fig. 3). However, we assume that the fluorescing substance (at 460 nm) that appeared during HpD photodestruction together with Trp may be different from those observed in a simple aqueous solution. In this case, fluorophore-460 is probably a photoproduct corresponding to the HpD-photosensitized destruction of Trp, since Trp had no significant effect on sensitizer photobleaching (data not shown). On the other hand, as shown in Fig. 4, the formation of photoproduct-460 is closely related to the magnitude of the HpD-sensitized photodegradation of this amino acid. Hence, it could be supposed that the nature of photoproduct-460 is different

from that of fluorophore-460, which was observed by other investigators [21] during self-sensitized photooxidation of Y-HpD in tumour cells. We suppose that in our conditions photoproduct-460 consists probably of formylbiliverdins, which form as a result of a photochemical reaction of the splitting of the tetrapyrrolic ring. It has been shown that certain metalloporphyrins are photooxygenated to yield formylbiliverdins as primary products [22]. Moreover, biliverdins are well known to degrade rapidly in the presence of $^1O_2$ sensitizers [23].

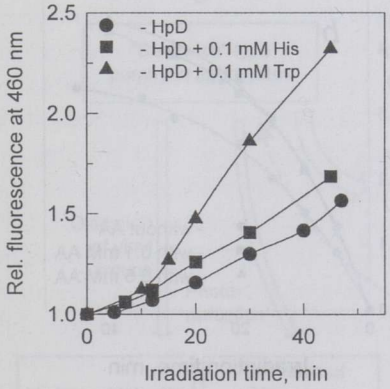

Fig. 3. Influence of histidine (His) (C_0 = 0.1 mM) and L-tryptophan (0.1 mM) on photoproduct-460 (λ_{ex} = 400 nm) formation during HpD illumination in water medium.

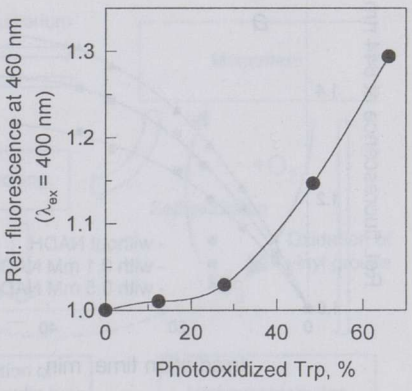

Fig. 4. Interrelation of photoproduct-460 formation and the degree of L-tryptophan (0.1 mM) oxidation (in 10 mM Tris-HCl buffer, pH 7.4) under HpD (10 μg/ml) photoexcitation.

An analysis of the spectral changes detected during the illumination of HpD shows that two main photoprocesses take place in aqueous solutions: photobleaching and photoproduct-644 formation. The dependencies of photoproduct-644 formation and photobleaching of HpD on the illumination time are shown in Figs. 1 and 2. As shown in Fig. 2, photoproduct-644 formation reaches saturation, whereas photobleaching continues. It is well known that tetrapyrrolic rings including chlorophylls and porphyrins are excellent electron donors either in their ground or excited states [24, 25]. Therefore, some researchers suppose that photoproduct-644 is a chlorin or chlorin-porphyrin linked system formed primarily from aggregates of HP-like porphyrins by photoreduction–photooxidation in a predominantly anaerobic environment [26]. These photoproducts have an absorption maximum around 640 nm (Fig. 1*c*). This may play an important role in HpD–PDT of cancer because of an increased absorbance in the spectral region of higher tissue transmission. The photoreduction reaction and, consequently, the formation of photoproduct-644 may be stimulated by adding a

suitable proton donor. Our data show that the rate of photoproduct-644 formation from HpD increased substantially in the presence of NADH (Fig. 5*a*) and L-ascorbic acid (AA) (Fig. 5*b*). A weak acceleration of the photoproduct-644 formation was observed also during illumination of HpD jointly with D,L-buthionine-[S,R]-sulphoximine, a low-toxicity inhibitor of glutathione biosynthesis (Fig. 5*c*). Moreover, NADH, AA, and BSO had no significant effect on the rate of HpD photobleaching (data not shown).

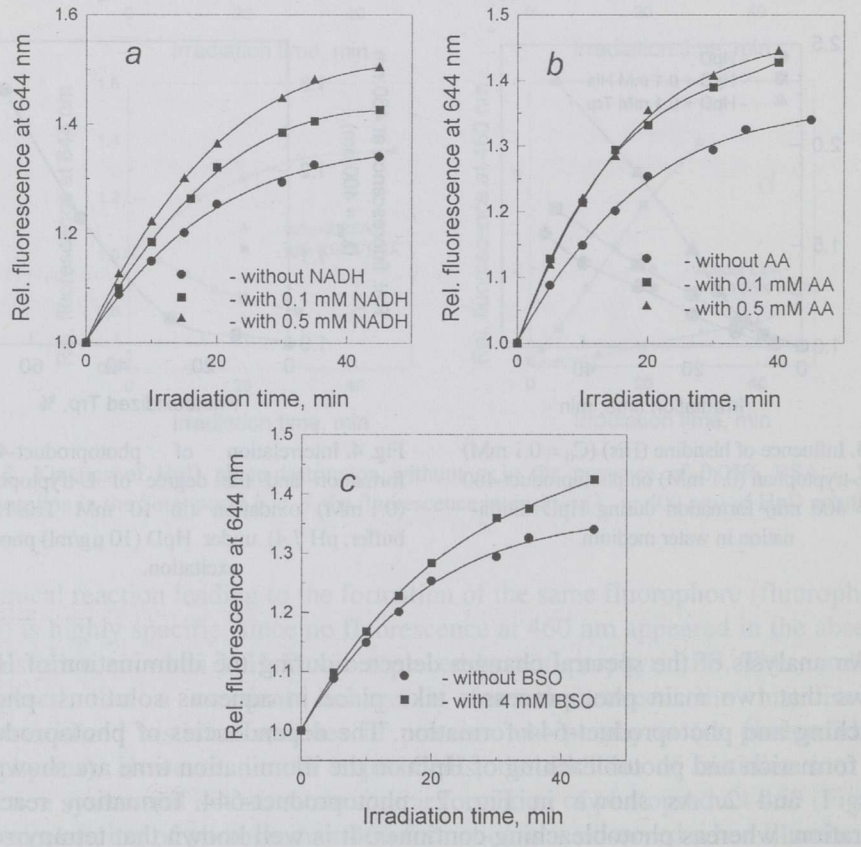

Fig. 5. Effectiveness of photoproduct-644 formation (estimated as changes of fluorescence at 644 nm under excitation at 400 nm) during HpD irradiation in the presence of NADH (*a*); ascorbic acid (AA) (*b*); and D,L-buthionine-[S,R]-sulphoximine (BSO) (*c*).

The results obtained are in good agreement with the data of other researchers who consider that the photobleaching of some tetrapyrroles, such as HP, HpD, PP, etc., is mediated by self-photogenerated $^1O_2$ that attacks the sensitizer macrocycles at the methine bridges to give pyrrole derivatives that do not absorb strongly in the visible light region and that do not act as a PS (Fig. 6) [23, 27].

Nevertheless, the formation of the HpD-photoproducts, absorbing light in the red spectral region (probably in consequence of type I processes), has been also detected. In addition, these photoproducts may have photodynamic activity. Indeed, Roberts et al. [28] performed a cell clonogenicity assay comparing photodegraded and fresh PII on CHO cells. The photodegraded PII exhibited significant phototoxicity although the fluorescence was bleached by more than 70%.

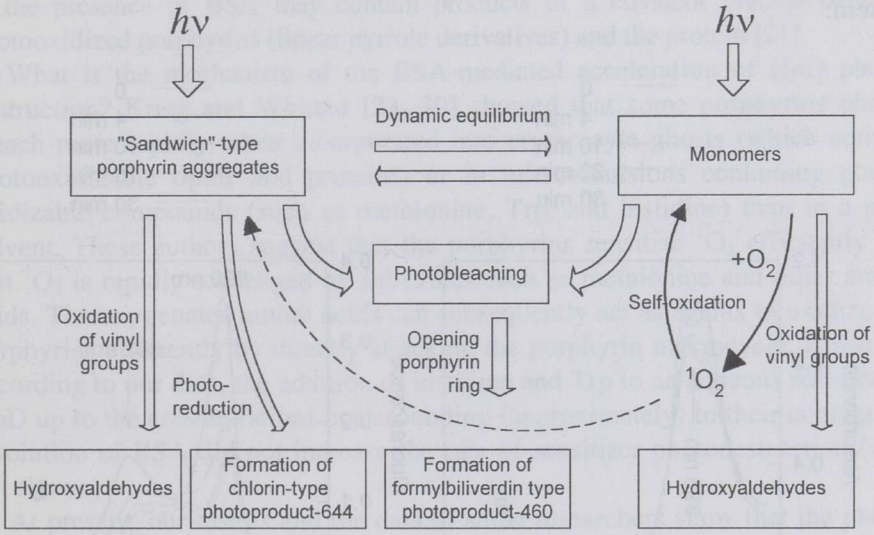

Fig. 6. Scheme of possible phototransformations of HpD in aqueous solution.

Self-sensitized photodestruction of HpD in the presence of serum albumin

As shown in Fig. 7, the binding of the HpD-components with BSA (in water medium) led to a change in the absorption and in the fluorescence emission spectra of this PS. In particular, long-wave shifts in the position of the Soret peak (from 370 to 395 nm) and the fluorescence emission peaks at 614 and 676 nm (about 6 nm) were observed. The data presented in Fig. 2 indicate that the rate of HpD photobleaching is strongly enhanced by the presence of BSA, which was accompanied with a considerable decrease in the porphyrin absorption in the Soret band as well as in the fluorescence emission peaks at 620 and 682 nm (Fig. 7). In 0.05% BSA (Fig. 2*a*), an about 2-fold increase of the initial rate of HpD photobleaching was observed when a fall of the porphyrin absorption in the Soret peak was measured. At the same time (Fig. 2*b*), a large (approximately 10-fold) rise of the initial rate of HpD photodestruction was observed when a fluorescence emission (at 620 nm) in the sensitizer solution was measured. Figure 2 demonstrates that after 5 min of light exposure of HpD in the presence of BSA already a 7% decrease in the porphyrin absorption in the Soret band was accompanied with a huge (about 50%) loss of the sensitizer fluorescence at

620 nm. Since the fluorescence quenching does not require cleaving of the tetrapyrrolic ring, strong intermolecular interactions must take place. Because serum albumin is susceptible to photosensitized modification by $^1O_2$ [29], we suppose that the anomalous fluorescence behaviour of HpD solution (during its illumination) may be conditioned by conformational changes in the protein. Therefore, evaluation of the porphyrin absorption is a more exact procedure for studying the photobleaching behaviour of HpD in the presence of a porphyrin-binding protein.

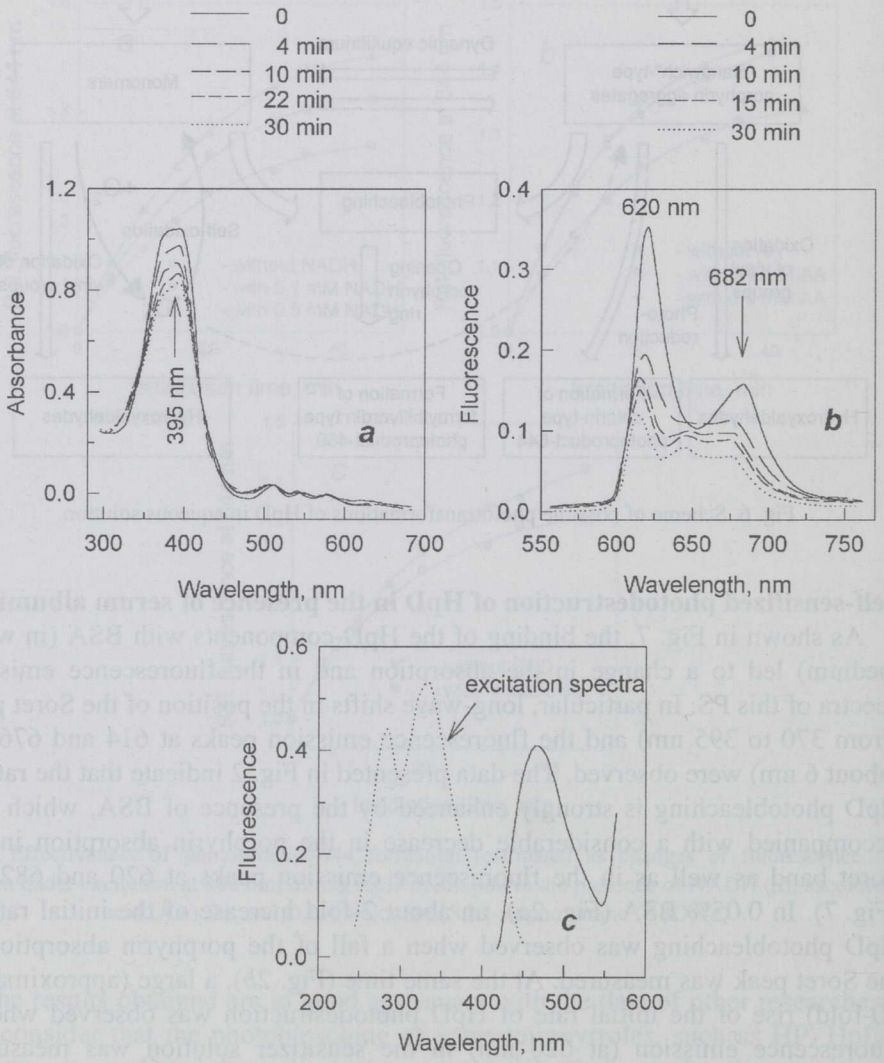

Fig. 7. Absorption (*a*) and fluorescence (λ_{ex} = 400 nm) spectra (*b*), of non-illuminated or illuminated HpD in 0.05% BSA solution; (*c*) fluorescence (λ_{ex} = 394 nm) and excitation (λ_{em} = 460 nm) spectra of photoproduct-460.

The photobleaching of HpD, mediated by BSA, was associated with a sharp decrease of the sensitizer fluorescence at 644 nm while the fluorescence of the HpD solutions at 460 nm increased (Figs. 2 and 7). Hence, the binding of the HpD components with BSA leads to an acceleration of the photoproduct-460 formation and, on the contrary, to a substantial inhibition of the photoproduct-644 formation. We suggest that photoproduct-460 consists of formylbiliverdins. However, the fluorescing substance (at 460 nm) that appeared during the HpD photodestruction in the presence of BSA may contain products of a covalent linkage between photooxidized porphyrins (linear pyrrole derivatives) and the protein [21].

What is the mechanism of the BSA-mediated acceleration of HpD photodestruction? Krieg and Whitten [23, 30] showed that some porphyrins photobleach more rapidly when incorporated into erythrocyte ghosts (which contain photooxidizable lipids and proteins) or in microemulsions containing photooxidizable compounds (such as methionine, Trp, and histidine) than in a pure solvent. These authors suggest that the porphyrins sensitize $^1O_2$ efficiently but that $^1O_2$ is rapidly scavenged by substrates such as methionine and other amino acids. The oxygenated amino acids can subsequently act as agents to oxidize the porphyrins efficiently by directly attacking the porphyrin macrocycle. However, according to our data, the addition of histidine and Trp to an aqueous solution of HpD up to the concentrations corresponding (approximately) to their contents in a solution of BSA did not increase the rate of sensitizer photodestruction (data not shown).

At present, our results and the data of some researchers show that the photobleaching yields and the mechanisms of HpD photodestruction may depend on its aggregate states. So, HSA had no significant effect on L-aspartyl chlorin e_6 photobleaching [31], which is a less hydrophobic sensitizer than HpD or PII. Photodegradation is enhanced with the addition of BSA probably due to a deaggregation of HpD, which slightly (about 20%) enhances absorption at the wavelengths of light source (405±20 nm). However, another mechanism may occur. It could be supposed that the binding of HpD to serum albumin, leading to monomerization of the aggregated PS molecules, was accompanied with an increase of the quantum yield of the HpD triplet state and, consequently, the formation of destroying porphyrins $^1O_2$. So, the HP monomer, which is the principal species present at high methanol content, has a triplet state yield of 0.93 [18]. By contrast, HP water solution containing aggregates had a lower triplet state yield, for example 0.56. As shown in Figs. 2 and 7 no fluorescence band at around 640 nm appeared during HpD photodestruction in the presence of BSA. The reason may be that complexation of the sensitizer in BSA leads to the disruption of "sandwich"-type porphyrin aggregates in which, presumably, the formation of photoproduct-644 takes place (Fig. 6). Indeed, Rotomskis et al. [26] showed that photobleaching is also observed in ethanol solution of HpD, where the monomeric state of the PS is dominant, although no absorption band at around 640 nm is detected after irradiation.

It is well known that NADH, Trp, AA, and histidine are very efficacious scavengers of $^1O_2$ [32]. However, in our experiments these biomolecules (up to 0.5 mM) and histidine at a concentration of 1.0 mM (data not shown) had weak effects on the rate of HpD photobleaching. Nevertheless, this may not rule out the involvement of $^1O_2$ in the photobleaching process. It has been proposed [33] that $^1O_2$ is generated very close to the triplet PS molecule (a complex is formed between the triplet state of the pigment and ground state of oxygen). As a result, the $^1O_2$ molecule may have a much higher probability of reacting with the sensitizer molecule that generated it than with the scavenger in the solution.

Thus, a comparison of the kinetics and the photoproducts formed at self-photosensitized oxidation of HpD without or with BSA indicates that the binding of HpD components in BSA strongly raises the intensity of photochemical processes with participation of $^1O_2$, leading to the splitting of the porphyrin macrocycle.

Influence of illumination on the stability of the HpD–BSA complex

The binding of HpD with BSA led to a long-wave shift in the position of the fluorescence emission peaks from 614 to 620 nm and from 676 to 682 nm (Fig. 7*b*). On the contrary, during illumination of HpD jointly with BSA already a short-wave shift in the position of these fluorescence emission peaks was observed (Fig. 7*b*). This phenomenon indicates that the photosensitized degradation of BSA molecules may lead, presumably, to the release of the HpD components from their complex with the protein.

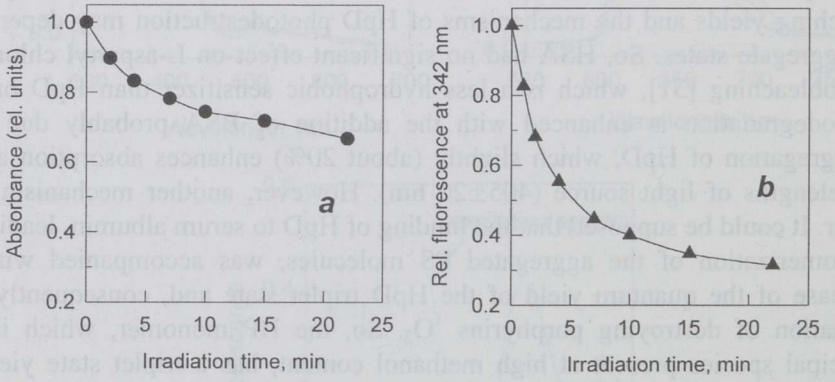

Fig. 8. Kinetic HpD-sensitized (C_0 = 10 μg/ml) photodestruction of BSA (initial concentration 0.1%) in aqueous solution (supplemented with 10 mM sodium phosphate buffer, pH 7.4): *a*, alteration of UV-absorbance (at 280 nm); *b*, fluorescence intensity at 342 nm (λ_{ex} = 280 nm) of BSA solution.

An analysis of the spectral changes detected during the illumination of BSA shows that this protein photodegrades rapidly in the presence of HpD. The results, given in Fig. 8*b*, show that the fluorescence of BSA at 342 nm strongly

decreased with the illumination time. Hence, the HpD-photosensitized oxidation of Trp residue in BSA may occur, since the fluorescence of this protein is known to be emitted mainly from its Trp residue [9]. In addition, the HpD-sensitized destruction of other amino acid residues in BSA (such as tyrosine and phenylalanine) also took place, because a substantial decrease of the protein absorption at 280 nm was observed (Fig. 8*a*). It could be supposed that the porphyrin binding sites in serum albumin may be very susceptible to HpD-photosensitized destruction. In fact, the quenching of Trp fluorescence in HSA by PII indicates that at porphyrin binding locus this PS is situated close to the Trp residue [7].

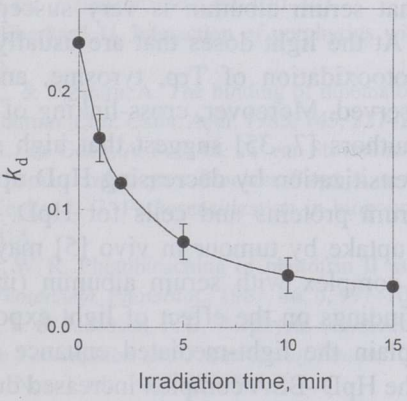

Fig. 9. The change of the coefficient of the distribution of HpD between 1-octanol and 0.2% BSA solution (k_d) during the illumination of the HpD–BSA system. Bars = SE.

Surprisingly, the HpD-sensitized degradation of BSA did not lead to a release of the HpD components from their complex with the protein. In contrast, under illumination the affinity of BSA to HpD enhanced. It was expressed (Fig. 9) as a considerable decrease of the distribution coefficients of HpD between 1-octanol and 0.2% BSA solution (k_d) with the illumination time. A common lowering of the sensitizer concentration (in consequence of its photodestruction) may be a cause of the k_d fall, since this alteration in the HpD–BSA system may cause a decrease of the unbound to BSA HpD fraction, which extracts from the BSA solution by 1-octanol better than the protein-bound. However, after 5 min of light exposure, when only 10% of the sensitizer had bleached (data not shown), already a 3-fold lowering of the k_d magnitude was observed (Fig. 9). A plausible explanation seems to be that the fall of k_d during the illumination of the HpD–BSA system is the result of HpD-induced photopolymerization of the protein molecules, which may put the brakes on the sensitizer diffusion. Indeed, marked porphyrins-sensitized photopolymerization of several proteins (in consequence of cross-linking) was observed previously by other investigators [8, 34]. Further,

suppose that increased stability of the HpD–BSA complex is the result of photochemical processes leading to covalent linking of certain HpD components (containing vinyl groups that are readily oxidized by $^1O_2$ to aldehydes) with the protein molecules. It is known that the biologically active components of HpD contain vinyl groups [1]. Moreover, the formation of hydroxyaldehydes during the self-sensitized photooxidation of PP has also been reported [22, 23].

Thus, we suppose that the photopolymerization of BSA molecules and the covalent binding of certain HpD components with the protein are the main factors responsible for the k_d fall observed during the illumination of the HpD–BSA system.

Our work showed that serum albumin is very susceptible to HpD-photosensitized modification. At the light doses that are usually used in the PDT of tumours, substantial photooxidation of Trp, tyrosine, and, probably, phenylalanine residues was observed. Moreover, cross-linking of BSA molecules may also take place. Some authors [7, 35] suggest that high serum concentrations result in reduced photosensitization by decreasing HpD uptake, since there is a competition between serum proteins and cells for HpD. Therefore, the light-induced increase in PII uptake by tumour in vivo [5] may be the result of the release of PS from its complex with serum albumin (in consequence of its photodestruction). Our findings on the effect of light exposure upon the HpD–BSA system cannot explain the light-mediated enhance of PII accumulation, because the stability of the HpD–BSA complex increased during illumination.

CONCLUSION

Our results may be useful for the development of improved HpD photoirradiation therapy. They show that at the illumination of HpD several photoprocesses take place: photosensitized destruction of the porphyrin macrocycle and photochemical modifications leaving the porphyrin ring intact (the formation of a chlorin-type molecule). The latter may play a very important role in HpD–PDT, since the formed photoproducts have light absorption in the red spectral region (around 640 nm). It was established that the mechanisms of HpD photodegradation are closely related to the aggregation state of this sensitizer. The quantum yields of HpD photobleaching may be considerable both in tumour loci and skin, because serum albumin can enhance the rate of HpD self-sensitized photooxidation. The formation of covalent links between HpD and serum albumin molecules may occur during the sensitizer photoexcitation. However, this assumption will be the subject of further investigation.

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HEMATOPORFÜRIINI DERIVAADI JA ALBUMIINI KOMPLEKSI FOTOOKSÜDATSIOONI UURIMINE

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Hematoporfüriini derivaadi (HpD) vesilahuse (pH 7,4) kiiritamine valgusega ($\lambda_{max} = 405 \pm 20$ nm; intensiivsus 27,5 mW/cm^2) tõi kaasa porfüriini makrotsükli

fotosensibiliseeritud destruktsiooni, mille tagajärjel vähenesid lahuse valguse absorptsioon ja fluorestsents, ning sellised fotokeemilised muutused, mis jätsid porfüriini tsükli terveks. On registreeritud 644 nm neeldumismaksimumiga fotoproduktide teke. Arvatavasti on fotoprodukt-644 kloriini tüüpi molekul, mis moodustub eelistatult porfüriinide agregaatidest konkureerivate fotooksüdatsiooni- ja fotoreduktsiooniprotsesside tulemusel. NADH ja askorbiinhape (prootonite doonorid) suurendasid märgatavalt fotoprodukt-644 teket, kuid härja seerumi albumiini (BSA) lisamine HpD vesilahusele pärssis seda. 0,05% BSA lisamine suurendas kahekordselt HpD fotolagunemise algkiirust, mida hinnati porfüriini neelduvuse vähenemise järgi Soret' ribas. Autorite arvates kaasneb HpD seostumisel BSAga fotosensibilisaatori agregeeritud molekulide monomeriseerumine, see omakorda suurendab HpD tripletse oleku kvantsaagist ja tõhustab singletse hapniku teket. Viimane on võimeline lagundama porfüriine. BSA on väga tundlik HpD fotosensibiliseeritud muutuste suhtes. Kasvajate fototeraapias tavaliselt kasutatavate valgusdooside puhul toimus oluline trüptofaani ja türosiini fotooksüdatsioon. Katsete põhjal selgus, et kiiritamine suurendab HpD ja albumiini kompleksi püsivust. Sellele viitavad katsed, kus HpD jaotuskoefitsient 1-oktanooli ja albumiini vahel langes võrdeliselt valgusdoosiga. On arutletud selle nähtuse mehhanismi üle.