

## Variation in the fluorescence decay properties of haematoporphyrin derivative during its conversion to photoproducts

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(Received March 25, 1990; accepted April 8, 1990)

**Keywords.** Porphyrins, photobleaching, photoproduct, singlet oxygen, fluorescence decay, triplet lifetime.

### Abstract

Haematoporphyrin derivative (HpD) photoproducts are formed in aqueous solutions during light exposure in the presence of oxygen. The evaluation of the fluorescence decay of the photoproduct-enriched HpD solution shows an increase in the short-lived components, especially about 2 ns, in comparison with HpD without photoproducts. The bleaching of the HpD fluorescence and the photoproduct formation by the fluorescence-exciting radiation has to be taken into account in the evaluation of stationary as well as time-resolved fluorescence measurements.

### 1. Introduction

During light exposure the photosensitizer haematoporphyrin derivative (HpD) changes its fluorescence and absorption properties because of photobleaching and photoproduct formation as shown in *in vitro* and *in vivo* measurements [1-5]. The bleaching of the fluorescence and the formation of the photoproducts depend on the presence of oxygen [6]. Whereas the absorbance of irradiated HpD solutions in the UV (Soret band) and the short-wavelength visible range decreases, the integral absorption in the red spectral region increases and a new maximum at about 640 nm is formed [7]. This increased absorbance in the region of high tissue transmission is of interest for photochemotherapy. The photoproducts are also photodynamically active; however, the activity is lower than that of HpD [7]. This paper presents some characteristics of the photoproducts, especially their formation. Most of the results are obtained by means of time-resolved fluorescence measurements.

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## 2. Materials

### 2.1. Chemicals

The HpD with a high content of aggregates, especially porphyrin dimers, used in these experiments was kindly provided by Dr. Lotz, Halle University. Its preparation, the chromatography results, its photodynamic activity and accumulation behaviour have been reported elsewhere [8, 9]. The photo-products were formed by laser irradiation (514 nm radiation of an argon ion laser ILA 120 from Carl Zeiss, Jena) or irradiation with a mercury lamp (HBO 200, Narva, Berlin).

The metronidazole was produced by KRKA, Yugoslavia (trade name, Efloran).

### 2.2. Experimental set-up

Stationary fluorescence spectra were measured by means of an optical multichannel analyser (OVA 284, Centre of Scientific Instruments, Berlin). The 514 nm line of an argon ion laser was used as excitation source. *In vitro* and *in vivo* fluorescence decay measurements of HpD were performed by the method of time-correlated single-photon counting (TCSPC) using a mode-locked argon ion laser ILA 120 equipped with an acousto-optic mode locker (514.5 nm; pulse separation, 8.1 ns) as the source of excitation pulses. The set-up for time-resolved detection of fluorescence after passage through a monochromator ( $\Delta\lambda = 11$  nm) consists of the TCSPC system SPC 100 (ZOS, Berlin) with the photomultiplier ELU 18 FM (U.S.S.R.). Deconvolution of the measured fluorescence curves was done by means of the program entitled "Global deconvolution of fluorescence kinetics". For more details see refs. 10–12.

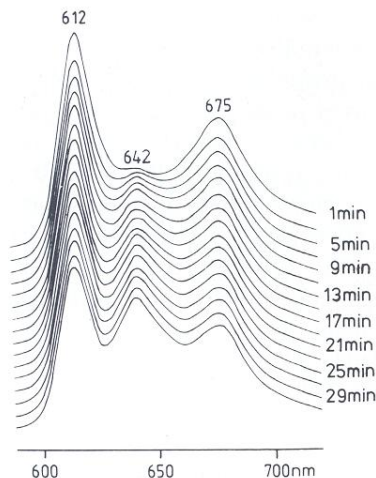
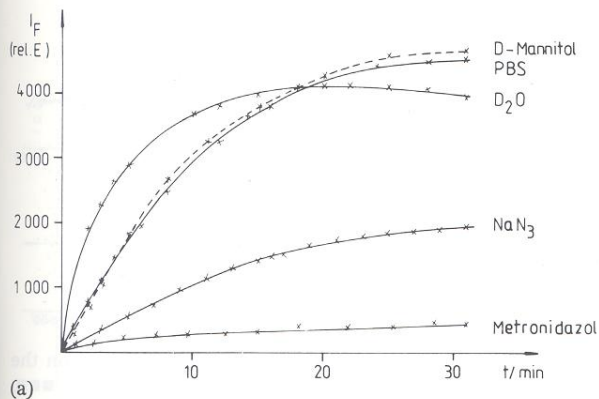
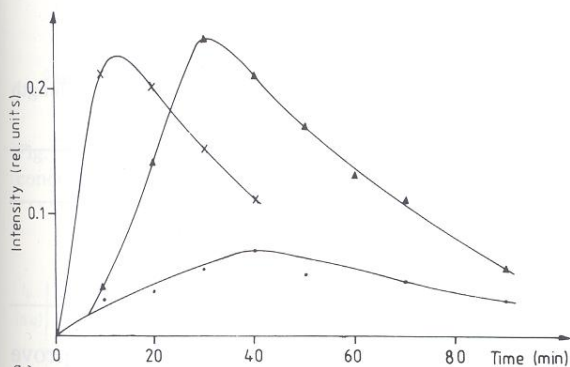


Fig. 1. Modification of the fluorescence spectra of a 0.1 mM HpD-NaCl solution during light exposure (514 nm; 100 mW; 1 ml; stirred).



(a)



(b)

Fig. 2. Formation of the fluorescent photoproducts of a 0.1 mM HpD-phosphate-buffered saline solution (pH 7.4) where the photoproduct fluorescence intensity at 642 nm was obtained after correction (subtraction of the value of monomer fluorescence at that wavelength fluorescence intensity of the photoproduct at 642 nm is dependent on the irradiation time for oxygen-, air- and argon-saturated solutions). (a) Dependence on the presence of 10 mM D-mannitol, 10 mM  $\text{NaN}_3$  and 0.5% metronidazole and the photoproduct formation in  $\text{D}_2\text{O}$ ; (b) dependence on the oxygen content of the solution ( $\times$ ,  $\text{O}_2$ ;  $\blacktriangle$ , air;  $\bullet$ , argon). The same irradiation conditions were used as for Fig. 1.

### 2.3. Animals

For the *in vivo* measurement a male ICR mouse (Institute of Cancer Research) was used bearing a solid subcutaneous Ehrlich carcinoma which was induced by injection of 0.2 ml Ehrlich ascites cells ( $5 \times 10^6$  cells  $\text{ml}^{-1}$ ).

## 3. Results

Figure 1 shows the formation of a new fluorescence band of HpD in aqueous solutions at around 642 nm during irradiation at 514 nm. Owing to photochemical reactions, various photoproducts are formed. This formation

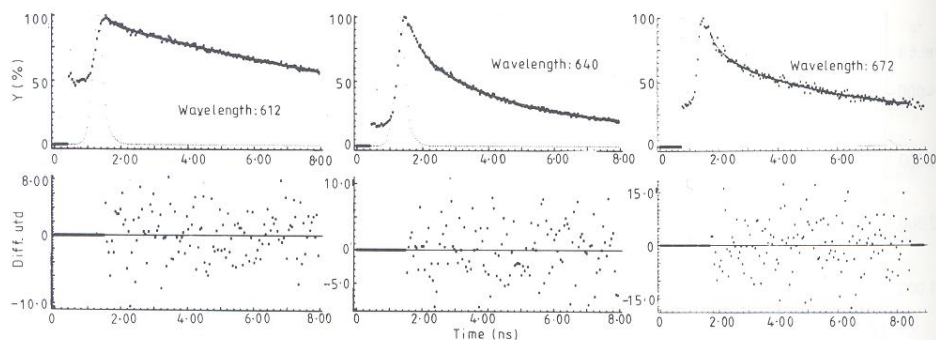


Fig. 3. Dependence of fluorescence decay curves of a 0.1 mM aqueous HpD solution on the fluorescence wavelength (excitation at 514 nm and  $10 \text{ mW cm}^{-2}$ ).  $\cdots$ , Laser pulse;  $\blacksquare$ , experimental values; upper diagrams, normalized intensity *vs.* time; lower diagrams, weighted residuals *vs.* time.

TABLE 1

Dependences of fluorescence decay times and normalized amplitudes on the wavelength ( $\tau_1 = 11\text{--}13 \text{ ns}$ ;  $\tau_2 = 2\text{--}3 \text{ ns}$ ;  $\tau_3 = 100\text{--}300 \text{ ps}$ )

$\lambda$ (nm)	$A_1$	$A_2$	$A_3$
612	0.99	—	0.01
640	0.69	0.25	0.06
672	0.86	0.10	0.04

depends on the presence of oxygen within the solution [6]. In order to prove whether ground-state or an activated form of oxygen plays the major role in the process of photoproduct formation, several scavengers were added to the HpD solution. As shown in Fig. 2(a), the singlet oxygen scavenger  $\text{NaN}_3$  significantly reduces the photoproduct formation, whereas the use of  $\text{D}_2\text{O}$  as solvent accelerates the process. This can be explained by the longer lifetime of singlet oxygen in  $\text{D}_2\text{O}$  ( $58 \mu\text{s}$ ) compared with  $\text{H}_2\text{O}$  ( $4.4 \mu\text{s}$ ) [13]. The  $\text{OH}^-$  scavenger D-mannitol does not influence the rate of photoproduct formation. Furthermore, the presence of metronidazole avoids its formation. Metronidazole interacts with the triplet state of HpD-forming complex compounds [14]. Therefore it can be concluded that the photoproduct formation is the result of the interaction of oxygen with the triplet state of HpD. Singlet oxygen is formed and plays the major role in this reaction. The singlet oxygen is also responsible for the decrease in the photoproduct fluorescence because it mediates the photochemical modification or destruction of the fluorescent photoproducts. This can be concluded from the decrease in the photoproduct fluorescence when  $\text{D}_2\text{O}$  is used as solvent (Fig. 2(a)) in accordance with the observed bleaching of the fluorescent photoproducts in Fig. 2(b) [6].

Time-correlated single-photon counting measurements were carried out at first on fresh aqueous HpD solutions using a low excitation radiation

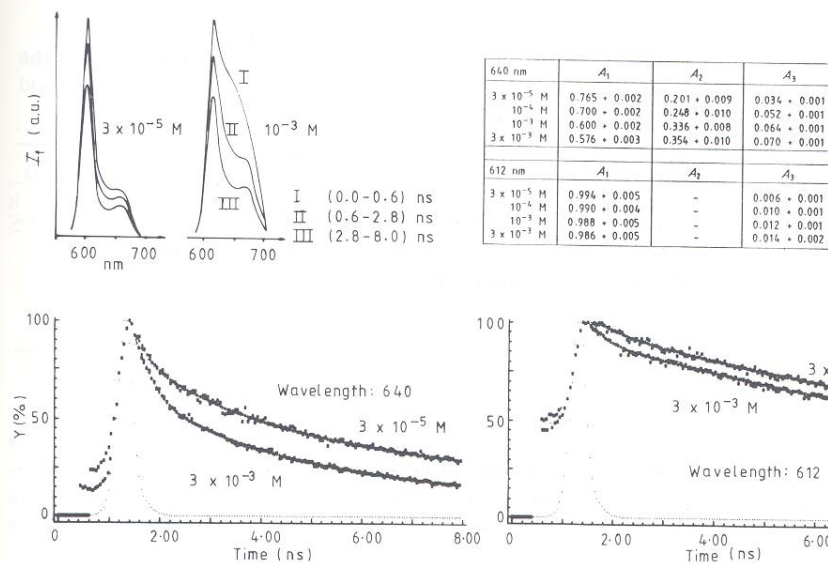


Fig. 4. Time-resolved spectra and decay curves of aqueous solutions of high and low HpD concentration and calculated relative amplitudes where:  $I_F$  is the average fluorescence intensity;  $\cdots$ , laser pulse;  $\blacksquare$ , experimental values.

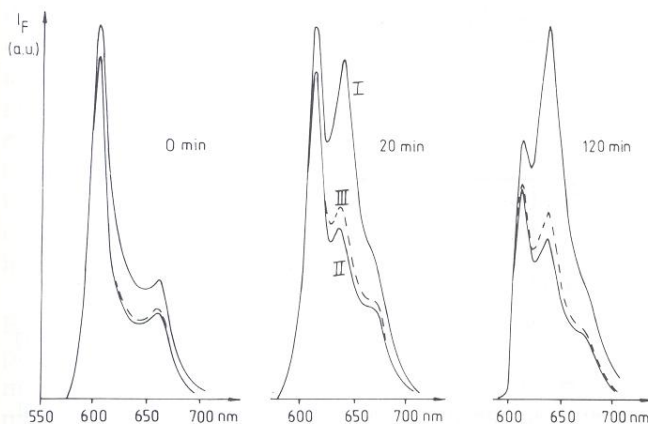


Fig. 5. Dependence of the time-resolved fluorescence spectra (average fluorescence intensity) on the irradiation dose (80 mW; 514 nm) where the time interval of the upper curve I is 0.1–1.0 ns, the time interval of the lower curve II is 1–7 ns and the time interval of the broken curve III is 0–7 ns ( $0 \text{ ns} = t(I_{F,\text{MAX}})$ ).

(10–20  $\text{mW cm}^{-2}$ ). Figure 3 shows decay curves at several fluorescence wavelengths. The faster relaxation at about 640 nm in comparison with the fluorescence maxima at 612 and 672 nm, should be noted. On the assumption of a decay of the form:

TABLE 2

Dependence of the relative changes in amplitudes of fluorescence decay components on the irradiation time (514 nm; 7 mW; stirred; 0.04 ml; the three decay times of 11 ns, 2.2 ns and 200 ps were fixed for calculation of the amplitudes)

Irradiation time (min)	$A_1/A_1 (t=0 \text{ min})$ (%)	$A_2/A_2 (t=0 \text{ min})$ (%)	$A_3/A_3 (t=0 \text{ min})$ (%)
0	100	100	100
2	99 ± 1	125 ± 2	84 ± 4
4	97 ± 1	129 ± 2	80 ± 4
6	90 ± 1	142 ± 2	87 ± 4

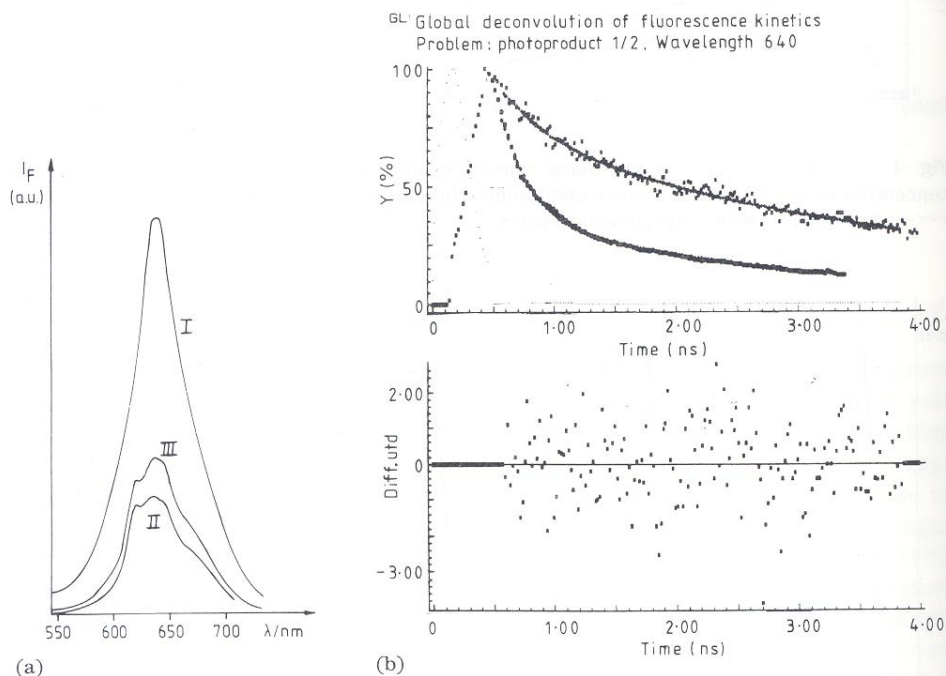


Fig. 6. (a) Time-resolved fluorescence spectra (upper curve I, 0.1–1.2 ns; lowest curve II, 1.2–7 ns; curve III, 0–7 ns) and (b) decay curves (channel width, 20.1 ps) with distribution of residuals of a strongly irradiated 0.1 mM HpD solution (800 J; HBO 200; 0.1 ml) in comparison with a weakly irradiated solution (20 J, upper curve). ·····, Laser pulse; ■■■, experimental values.

$$\sum_i A_i \exp\left(-\frac{t}{\tau_i}\right)$$

three fluorescent components of the HpD solution can be determined. Table 1 presents the results.

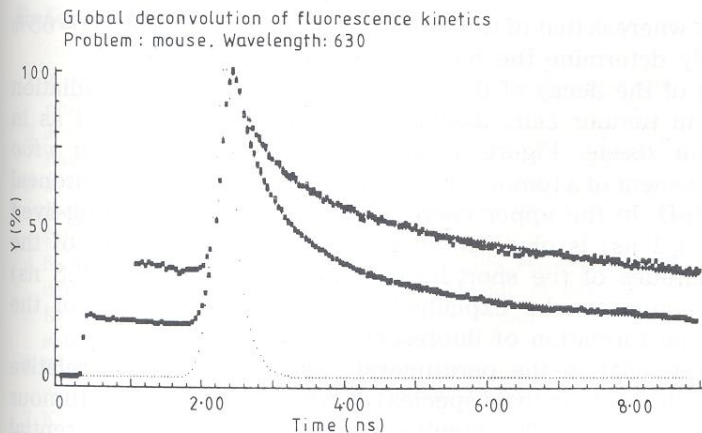


Fig. 7. *In vivo* decay curve of the 630 nm fluorescence of a HpD-labelled solid Ehrlich carcinoma (4 h after intraperitoneal administration of HpD; 20 mg per kilogram of body mass) where  $\tau_1 = 13 \pm 2$  ns,  $\tau_2 = 1.0 \pm 0.3$  ns and  $\tau_3 < 90$  ps (the  $\tau_3$  value results from scattered light which passed the dielectric filter reflecting the greatest part of the excitation radiation): upper curve,  $10 \text{ mW cm}^{-2}$ ,  $A_1 = 91.7 \pm 0.1$ ,  $A_2 = 5.6 \pm 0.1$ ,  $A_3 = 2.7 \pm 0.1$ ; lower curve,  $10 \text{ mW mm}^{-2}$ ,  $A_1 = 72.1 \pm 0.1$ ,  $A_2 = 20.0 \pm 0.1$ ,  $A_3 = 7.9 \pm 0.2$ . . . . ., Laser pulse; ■■■, experimental values.

Besides a dependence on the fluorescence wavelength, the decay curves depend on the HpD concentration as shown in Fig. 4. With increasing HpD concentration the amplitudes of the short-living components increase especially at around 642 nm. The reabsorption of fluorescence light did not have much influence on fluorescence spectra even for the highest HpD concentration, as was shown by using various excitation geometrics. These results allow us to conclude that the 11 ns component belongs to HpD monomers and the shorter components belong to several types of aggregated species. These conclusions were also drawn by other workers [15–17], whereas yet other investigators [18] reported that the 2 ns component belongs to the monomers.

Now we consider the fluorescence behaviour under intense irradiation. Besides a decrease in the 612 nm fluorescence the formation of fluorescent photoproducts can be observed. Time-resolved spectra were recorded simultaneously for three time intervals of the fluorescence decay: I, 0.1–1.0 ns; II, 1–7 ns; III, 0–7 ns. They show that the fluorescence at 642 nm has its strongest components in the shortest interval, whereas the long-living component belongs mainly to the monomer fluorescence at about 612 nm (Fig. 5). However, the same decay times of about 100–300 ps, 2–3 ns and 11–13 ns for the fluorescence of the sample containing photoproducts can be determined. There was no evidence for the presence of a fourth fluorescence component. Table 2 presents the change in the relative amplitudes during irradiation.

After an irradiation period of some hours with a mercury lamp (dose, 800 J; 1 ml; cross-section,  $1 \text{ cm}^2$ ) the contribution of the monomers decreases

to  $A_1 \approx 7\%$  (642 nm) whereas that of the short-lived components with  $A_2 \approx 65\%$  and  $A_1 \approx 28\%$  mainly determine the fluorescence behaviour (Fig. 6).

The shortening of the decay of the HpD fluorescence during irradiation can be found also in tumour cells incubated with HpD [12] as well as in HpD-labelled tumour tissue. Figure 7 shows the results of an *in vivo* fluorescence measurement of a tumour-bearing mouse 4 h after intraperitoneal administration of HpD. In the upper curve the dominance of the long-lived component ( $\tau_1 = 13 \pm 2$  ns) is obvious whereas with rising intensity of the excitation the amplitudes of the short-lived components ( $\tau_2 = 1.0 \pm 0.3$  ns) increase. This behaviour can be explained by the photobleaching of the monomers and by the formation of fluorescent photoproducts.

Further measurements in the peritumoral tissue show higher relative values of the amplitude  $A_1$  (long-lived species) in comparison with the tumour tissue (equal dose of exposure). This result could be explained by preferential accumulation of HpD aggregates, especially covalently bound dimers within tumour tissue [15]. However, a larger formation of the photoproducts within the tumour tissue must be additionally taken into account owing to the higher HpD concentration.

#### 4. Discussion

During intense light exposure, HpD forms fluorescent photoproducts. The photoprocess proceeds via the excited triplet state of porphyrins and depends on the irradiation conditions, on the HpD concentration and on the concentration of oxygen, especially on the presence of singlet oxygen [6] (the oxygen dependence is not commonly accepted in the literature [3]). The photo-induced modification of the absorption spectrum due to the photoproduct formation results in an increase in the integral absorption in the red spectral range [7] which is of interest in photochemotherapy. However, photoproducts show a lower photodynamic activity as confirmed by cell inactivation experiments [7]. Aqueous HpD-photoproduct enriched solutions exhibit a faster decay of the triplet states and a smaller yield of singlet oxygen formation than HpD solutions without photoproducts [19]. This should be the explanation for the lower photodynamic activity of the photoproducts.

The decrease in the singlet lifetime of HpD during irradiation as well as the results of the chromatography on Sephadex coarse of aqueous HpD solutions containing photoproducts in comparison with that of HpD aggregates (results not shown) allows us to conclude that the nature of the photoproducts is possibly connected with aggregated forms, especially photodimers.

As shown by time-resolved fluorescence measurements the photoproducts formed exhibit spectral and time characteristics similar to those typical of the HpD aggregates. In the interpretation of the results of fluorescence measurements of HpD-containing samples, *e.g.* HpD-incubated cell suspensions or HpD-labelled tumour tissue, one has to consider the photochemical transformation (photoproduct formation) of HpD induced by the fluorescence exciting radiation.



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