

Photobleaching of HpD Fluorescence and Formation of Photoproduct *In Vivo* and in Solution

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(Received September 9, 1989; in final form December 15, 1989)

The fluorescence spectrum of HpD-labelled tumors as well as of HpD in aqueous solutions changes during irradiation. The integrated fluorescence intensity decreases and a fluorescent photoproduct is formed. During light exposure the fluorescence of the photoproduct increases, reaches a maximum and decreases. The photoproduct formation as well as the fluorescence bleaching depend on small amounts of oxygen and do not occur in oxygen free solutions. In the absorption spectrum of irradiated solutions an increased absorption especially around 640 nm appears. Its formation is also oxygen dependent but this photoproduct is photochemically stable. It has a photodynamical activity below that of HpD.

KEYWORDS: *photobleaching, photoproduct formation, fluorescence, HpD*

INTRODUCTION

The fluorescence spectra of HpD-labelled tumors change during excitation. With increasing irradiation dosage a bleaching of the fluorescence is observed (e.g., [1]) as well as the formation of a new fluorescence peak located between the characteristic fluorescence peaks of HpD. These features are reported on the fluorescence spectra of HpD incubated cells [2] as well as of HpD-labelled animal tumors [3, 4] and human tumors [5]. This formation of a new tumor fluorescence located at about 660 nm in tissue is controversially interpreted, e.g., as a tumor specific fluorescence, as a result of HpD-tissue interaction or as a photoproduct.

The aim of this paper is to give more information about the modification of the fluorescence spectrum *in vivo* and *in vitro* under irradiation, especially the generation of a photoproduct and the conditions of its formation.

MATERIALS AND METHODS

Animals

The experiments were performed on male ICR mice with a subcutaneously induced solid Ehrlich carcinoma (injection of 7 day old Ehrlich ascites cells, 10^7 cells/ml).

Chemicals

HpD was kindly supplied by Dr. Lotz, Halle University. It was prepared from bovine blood and enriched with DHE-dimers by gel filtration chromatography on Sephadex G 25 coarse. Photofrin II (Photomedica, USA) and Hp (Porphyrin Products, Logan) were used as received.

Experimental Set Up

The *in vivo* fluorescence was excited with the 364 nm UV line of an argon-ion laser (ILA 190, CZ Jena). From this line the background of the argon laser discharge was separated by the use of two Brewster prisms [6]. An optical multichannel analyzer (OVA 284, ZWG Berlin) served as a fluorescence detector system. For the suppression of the scattered UV-radiation an additional dielectric filter reflecting the UV-radiation was used in the front of the detector. The spectra of solutions were registered by means of the absorption spectrometer specord MV 40 and the spectrofluorimeter Fica 55.

RESULTS AND DISCUSSION

The modification of the fluorescence spectrum of HpD-labelled solid Ehrlich carcinoma *in vivo* during irradiation is shown in Figure 1. The integral fluorescence intensity decreases with increasing irradiation time. At the same time a new fluorescence band located around 660 nm appears and grows with the irradiation time. The decrease of the integral fluorescence and the appearance of a new fluorescence peak can also be observed in an aqueous solution of Photofrin II, HpD and Hp at concentrations of 0.1 mM and higher. Figure 2 shows the modification of a 0.2 mM HpD aqueous solution under irradiation. In comparison, the *in vivo* spectrum (Fig. 1) is spectrally shifted approximately 20 nm due to the different environmental micropolarity of HpD in tissue (e.g., [7]), but the behavior, under irradiation *in vivo* and in aqueous solution is qualitatively the same. Therefore, this behavior is not tumor specific and can also be observed under higher HpD concentration (>0.2 mM) in aqueous solution of HpD. So, in the following, the processes are investigated in aqueous solution.

First we consider the intensity of the fluorescence peak located at 612–613 nm under irradiation. As shown in Figure 3, the fluorescence intensity $I^F(613 \text{ nm})$ decreases with increasing irradiation time. The decrease exhibits an exponential decay. As known from the fluorescence excitation spectrum in saline solution, the HpD fluorescence is mainly excited at 395 nm (monomer absorption). Figure 4 shows the modification of the HpD absorption under irradiation. An increased bleaching of the absorption with irradiation time in the Soret band is obvious. Therefore, the decrease of the integral HpD fluorescence results from the photochemical destruction of the HpD molecules, most likely from photooxidative conversion of the monomers into other forms (dimers or associates of different types). The photobleaching of HpD absorption and the decreasing of the fluorescence exhibit a strong dependence on the oxygen content in the solution [4]. As shown in Figure 3, the decrease of the 613 nm fluorescence is different in the case of oxygen and argon saturated solution. With increasing oxygen content the bleaching increases whereas a sample with high oxygen concentration in darkness does not show any effect.

Furthermore, the bleaching depends on the irradiation wavelength. The highest bleaching rate was obtained by irradiation in the Soret band, the lowest in the red spectral region. This indicates that the bleaching rate follows the absorption spectrum of HpD.

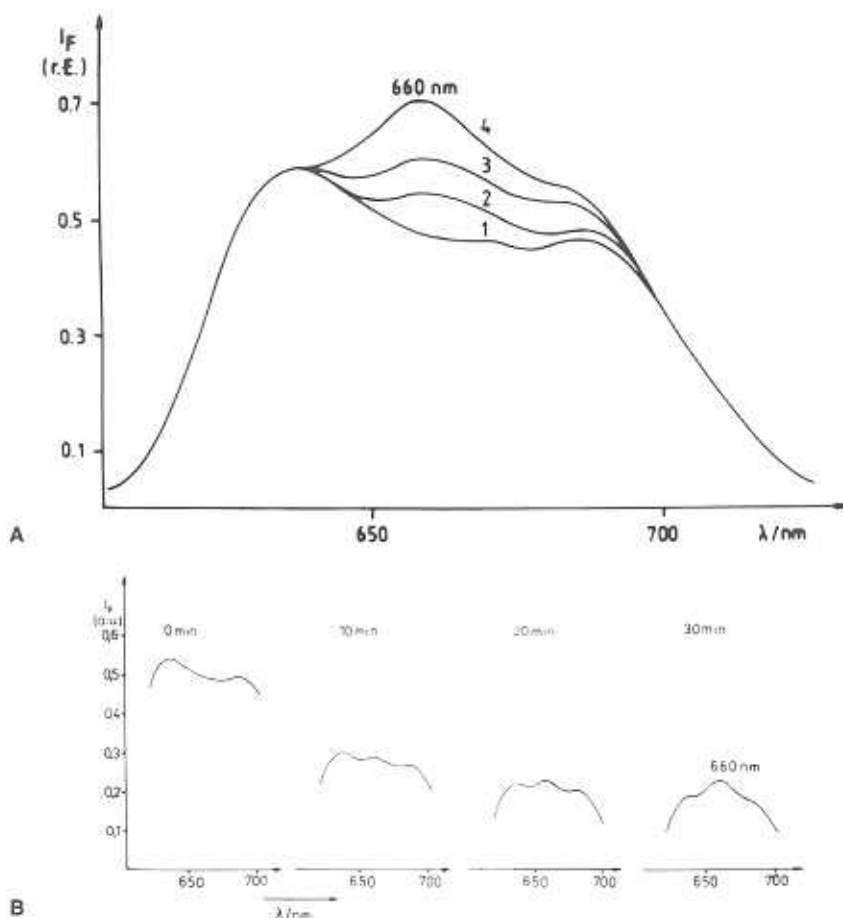


FIGURE 1 Modification of the *in vivo* fluorescence spectrum of a HpD-labelled solid Ehrlich carcinoma in mice (i.p., 10 HpD/kg body weight) during irradiation with 10mW laser light at 366nm (appr. 0.3W/cm²) at different irradiation times. 1: 0 min, 2: 10 min, 3: 20 min, 4: 30 min. A, Fluorescence spectra normalized to the 635 nm fluorescence peak. B, Absolute values of the decrease of fluorescence under irradiation.

It should be noted that by the strong photobleaching of the HpD fluorescence, every fluorometric measurement on HpD containing samples *in vivo* and *in vitro* can be influenced by the excitation radiation. Therefore this phenomenon must be taken into account.

Now we discuss the behavior of the fluorescence band located around 640 nm in solution. Figure 2 shows that the fluorescence intensity in this spectral region rises with increasing irradiation time in relation to the intensities of the 613 nm and 675 nm fluorescence bands.

The absolute value of the fluorescence intensity of this additional band around 640 nm is given by subtraction of the contribution from the HpD fluorescence at 640 nm (under consideration of bleaching of that fluorescence). This background-free fluorescence is depicted in Figure 5 for HpD solutions of different oxygen content. Dependent on this content, the fluorescence rises, reaches a maximum and slowly decreases. In order to get some insight into this behavior, the absorption and the fluorescence excitation spectrum was investigated. From the absorption spectra of Figure 4 it is obvious that no uniform bleaching

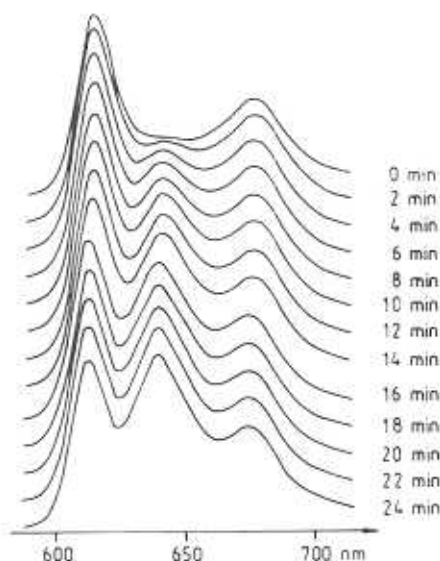


FIGURE 2 Modification of the fluorescence spectra of a 0.2 mM HpD-NaCl solution, air-saturated, wavelength of fluorescence excitation: 364 nm, 10 mW

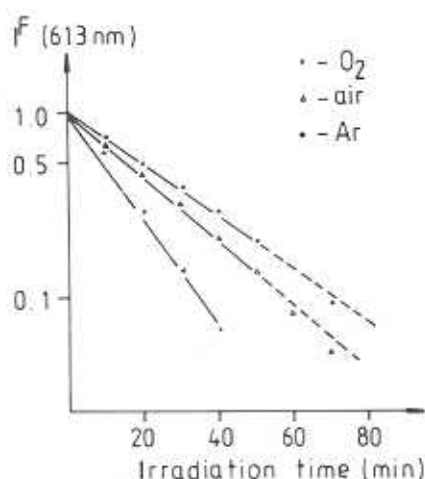


FIGURE 3 Decrease of the fluorescence intensity of a 0.1 mM HpD-NaCl solution at 613 nm after irradiation with a mercury lamp (50 mW/cm^2), wavelength of fluorescence excitation: 400 nm. I^0 for oxygen-, air- and argon-saturated solutions in logarithmic scale

of the absorption occurs. The absorption increases to a certain extent in the spectral region 450–500 nm and 600–700 nm, especially at 640 nm. The formation and the following stationary behavior of the background-free additional absorption at 640 nm is shown in Figure 6. The fluorescence excitation spectrum of the 640 nm fluorescence has maxima at 400 nm, 490 nm and in the region around 635 nm. From these results the formation of at least one photoproduct can be concluded.

At an irradiation time at which the fluorescence intensity of the photoproduct decreases

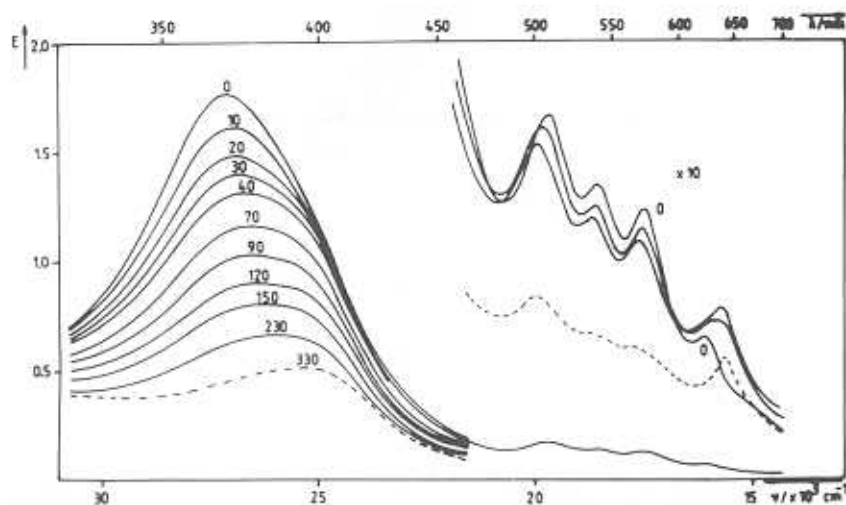


FIGURE 4 Absorption spectrum of a 0.1 mM HpD-NaCl solution in dependence on the irradiation time (in min.), the same irradiation conditions as in Figure 3 (solid line of the magnified curves: 0 min, 10 min and 20 min irradiation)

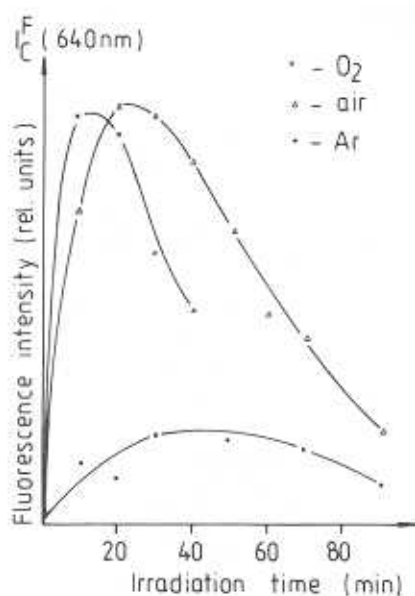


FIGURE 5 Fluorescence intensity of the photoproduct around 640 nm (corrected) in dependence on the irradiation time for oxygen-, air- and argon-saturated solutions,

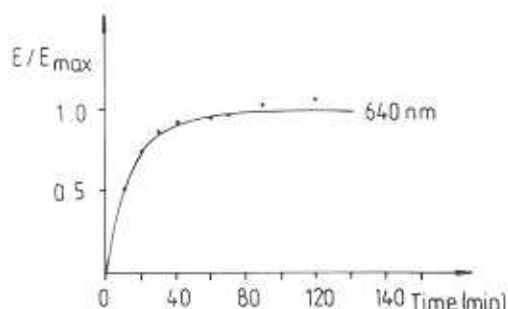


FIGURE 6 Relative absorbance of the photoinduced additional peak around 640 nm, the same irradiation conditions as in Figure 3.

($t > 25$ min for air-saturated solution, Figure 5) the absorbance around 640 nm seems to be constant (Fig. 6). Assuming this absorption band belongs to the fluorescent photoproduct with a fluorescent maximum around 640 nm (Fig. 2), then the decrease of its fluorescence in Figure 5 can be supposed to be by Foerster transfer between the monomer species (monomer fluorescence decreases also, see Fig. 3) and the photoproduct. For porphyrins a Foerster radius of approximately 2.5 nm can be estimated. However, the energy transfer rate in the case of a HpD concentration of 10^{-4} M is too small in comparison with the rate of monomer fluorescence. More likely, the additional absorption around 640 nm belongs to another photoproduct (or products) which is (are) photochemically stable, whereas the fluorescent photoproduct with the fluorescence maximum around 640 nm seems to be photochemically unstable. As shown in Figure 5 the decrease of the fluorescence as well as the formation of the photoproduct depends on the oxygen content.

The oxygen dependence of the photoproduct formation is not commonly accepted in the literature (e.g., [8]). In argon saturated solution this formation is less effective and needs a higher dose of irradiation (Fig. 5). In order to reduce the oxygen content further we prepared an "oxygen-free" solution by repeated evacuation and repeated input of helium, purity 99.99%. No photoproduct formation and fluorescence bleaching could be observed, monitoring the absorption around 640 nm as well as the fluorescence of the sample. Also at the extreme irradiation time of 10 h with 50 mW/cm^2 no photoproduct formation or bleaching were recorded. When the irradiated oxygen free solution was brought in contact with the atmospheric air, the photoproduct formation as well as the photobleaching of the HpD fluorescence could be observed by monitoring the fluorescence within 30 s. The formation of the photoproduct was also at pressures in the order of 10 Pa atmospheric air. Therefore, it should be possible to detect optically very low oxygen concentrations (in the order of 10^6 M).

This high sensitivity may be of interest for studying the oxygen status of tumor tissue by optical means. The preparation of investigations are underway.

The formation of the fluorescent photoproduct was found to be mediated via the excited triplet state of porphyrin. Quenching of the triplet state with the triplet quencher metronidazole inhibits the photoproduct formation. The singlet oxygen quencher NaN_3 appreciably reduces the formation. In the solvent D_2O , a medium with long singlet oxygen lifetime, the formation as well as the destruction of the fluorescent photoproduct runs faster than in air-saturated aqueous solution. These results suggest that singlet oxygen is responsible for photoproduct formation.

In relation to the HpD absorption at 625–630 nm used in the photochemotherapy, the absorption of the photoproduct is red shifted and stronger, see Figure 4. Therefore its photodynamic activity is of interest. For this purpose an irradiated HpD solution with a high content of photoproduct and a nonirradiated HpD solution were incubated in human erythrocytes and the devitalization (lysis) under irradiation was determined by scattering measurements. It turns out that the photoproduct also has a photodynamic activity but less than HpD at a wavelength shorter than 640 nm. At its absorption maximum around 640 nm, it is comparable with the low activity of HpD [9]. This reduced photodynamic activity was found to be in correlation with a reduction of the triplet lifetime of the photoproduct measured in an excite and probe experiment.

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