The suggested method thus provides information about the process of platelet aggregation which differs radically from that obtained by the standard turbidimetric method. This information will be very useful both for scientific research and for the diagnosis of changes in the aggregating activity of platelets during the development of various diseases.

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PHOTOGENERATION OF SINGLET MOLECULAR OXYGEN BY COMPONENTS OF HEMATOPORPHYRIN IX DERIVATIVE

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UDC 616-006.6-018.1-02:615.831.4]-07

KEY WORDS: singlet molecular oxygen; porphyrins; photodynamic therapy.

The method of photodynamic therapy (PDT) is based on photodestruction of tumors, the process being sensitized by molecules of porphyrins selectively accumulated in cancer cells [5]. The active fraction of "hematoporphyrin IX derivative" (HPD), known commercially as "Photofrin II," is the agent most widely used in clinical trials at the present time [15]. HPD is a complex mixture of variable composition of products of alkaline treatment of acetylated hematoporphyrin IX, and includes porphyrin monomers: hematoporphyrin IX (HP), hydroxyethylvinyldeuteroporphyrin IX, protoporphyrin IX, and their dimeric and oligomeric derivatives; the phototherapeutic effect, moreover, is determined by these di- and oligomeric fractions. Photofrin is relatively richer in polymeric fractions than HDP but also contains a mixture of the above compounds [5, 15]. Previous investigations [2, 3, 6, 8, 10, 11, 13] have shown that if solutions and cells stained with HP, HDP, and Photofrin are illuminated, excited oxygen molecules in the $^1\Delta_g$-state ($^1O_2$) which appear when energy is transferred to O_2 from triplet porphyrin molecules, are formed. This led to the idea that it is $^1O_2$ which is the principal cytotoxic factor responsible for the action of these preparations [5]. However, it must be noted that $^1O_2$ generation has been investigated only in specimens containing HP or the total of all components of HDP, whereas activity of the polymeric components, responsible for the phototherapeutic effect, has not been successfully investigated. The technique of separation and purification of monomeric, dimeric, and oligomeric components of HDP which have been developed [12] has made the experimental study of this problem possible.

In the investigation described below a direct luminescent method of recording singlet oxygen [9] was used to determine the efficiency of $^1O_2$ generation by dimeric (DF) and oligomeric (OF) fractions of HDP, and also by purified HP, under conditions simulating their state in cellular solutions and cell membranes.

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(Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 10, pp. 440-442, October, 1989. Original article submitted November 2, 1988.

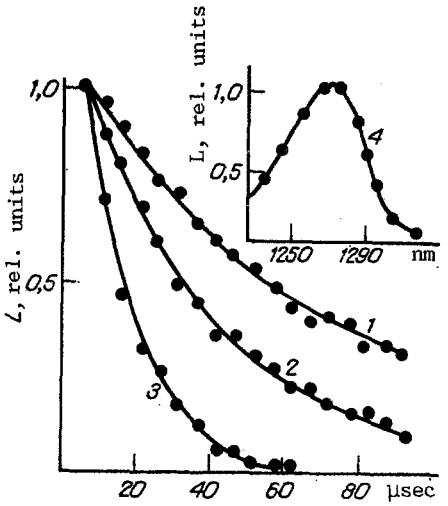

Fig. 1. Kinetics of extinction (1-3) and luminescence spectrum (4) of $^1O_2$, sensitized by HP in D_2O (1, 4), D_2O containing 2% Triton X-100 (2), and in ethanol (3). Kinetic curves averaged from calculation for 150,000 laser flashes. Width of monochromator slits was 8 nm.

EXPERIMENTAL METHOD

Luminescence of $^1O_2$ was excited by nanosecond pulses from an LGI-21 nitrogen laser (337.1 nm) and recorded in the 1270 nm region by means of a cooled FEU-83 photomultiplier through a high-transmission MS-80 monochromator (Central Design Office, Academy of Medical Sciences of the USSR). An NTA-1024 multichannel analyzer was used to store the FEU signal. The results were analyzed on an EMG-666 microcomputer (Hungary) [1, 10]. Relative values of quantum yields of $^1O_2$ generation (γ_Δ) by the porphyrins were determined by comparing the intensity of luminescence of $^1O_2$ in solutions of the test porphyrins with that in a solution of tetrasulfophenylporphyrin (TSPP) by the method described previously [2, 10] (Table 1). HP was obtained by the method in [7] (the hydrogen bromide concentration was 50%) and purified by column chromatography on silica-gel 40/100 (Reanal) in a system of chloroform–methanol–water (65:25:4). HDP was obtained from HP diacetate by treatment with 0.1 N NaOH solution for 1 h [15], followed by precipitation of acetic acid and drying in air. DF and OF were isolated from HDP by gel-filtration on Fractogel HW-40(s) in a column with dimethyl sulfoxide–acetic acid system (1:1 by volume). The DF and OF thus obtained were diluted with water, precipitated with sodium bicarbonate, centrifuged, and washed with water. The purity of the preparations was verified by gel-chromatography in the same system. The concentration of the basic substance in the HP preparations was not less than 95%. The DF contained not more than 1% of monomeric impurities, and no monomeric impurities whatever were found in the OF specimens. TSPP was obtained by sulfonation of tetraphenylporphyrin as in [14]. The porphyrins were dissolved in Na/K-phosphate buffer made up with deuterated water (0.04 M, pD 7.1-7.2), in buffer containing 2% of the detergent Triton X-100 (Loba-Chemie, Austria), or ethanol. Deuterated water (D_2O, produced by the Isotop Leningrad Medical Preparations Combine, D_2O content not below 99.9%) was used without additional purification, and the ethanol was purified by redistillation over calcium oxalate. To prepare aqueous solutions, crystals of porphyrin were dissolved in 0.5 ml of 2% solution of KOH in D_2O, and then diluted with buffer to the required optical density value at the excitation wavelength (0.1-0.3 OD). The concentration of porphyrins in the aqueous solutions was 30 μM for HP and 7-9 μM for DF and OF and in the alcoholic solutions it was 4-7 μM for all the compounds.

EXPERIMENTAL RESULTS

During laser excitation of the various porphyrins in air-saturated solutions photosensitized luminescence of $^1O_2$ was observed with a maximum at 1270 nm and with a life of 67 μsec in D_2O, 61 μsec in K/Na-phosphate buffer in D_2O, 38 μsec in buffer containing 2% Triton X-100, and 14 μsec in ethanol (Fig. 1).

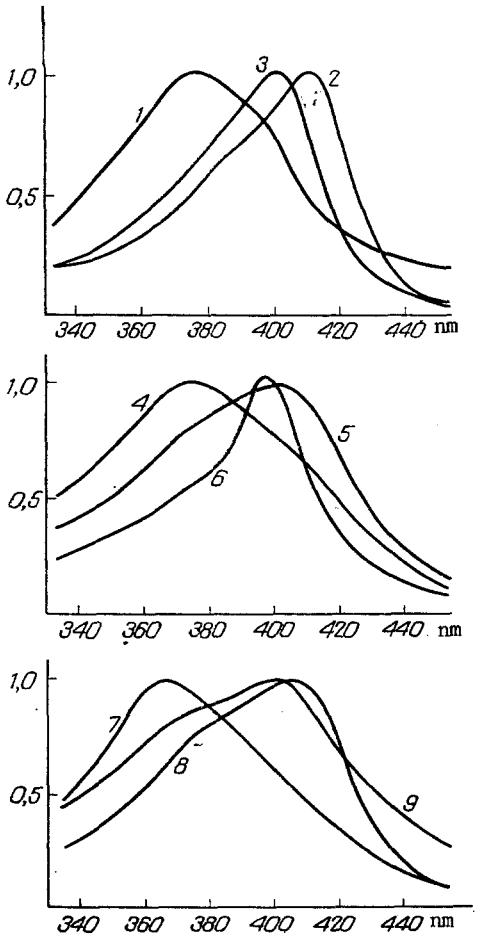

Fig. 2. Absorption spectra of solutions of HP (1-3), DF (4-6), and OF (7-9) in D_2O (1, 4, 7), D_2O containing 2% of Triton X-100 (2, 5, 8), and in ethanol (3, 6, 9).

Hematoporphyrin IX. Generation of $^1O_2$ by a preparation of hematoporphyrin dihydrochloride, manufactured by Koch-Light Laboratories (HPDHCl), was studied previously in the writers' laboratory by measuring oxygen luminescence [2, 10]. Comparison with purified HP obtained by the method described above shows that these two preparations are virtually indistinguishable in their spectral properties and photosensitizing activity (Fig. 2; Table 1). In agreement with previous data [2, 10, 11], we observed that the values of γ_Δ in alcoholic and detergent solutions are significantly higher than in detergent-free aqueous solutions (Table 1). Comparison of the absorption spectra shows that the principal peak of the Cope band (400-410 nm) in alcoholic and detergent solutions corresponds to monomeric HP molecules. In detergent-free aqueous solutions the principal peak (375 nm) corresponds to associated HP molecules [4]. Comparison of the data indicates that association of porphyrin molecules in water reduced the efficiency of $^1O_2$ generation. However, associated HP molecules do nevertheless exhibit moderate photosensitizing activity [2, 10].

Dimeric and Oligomeric HPD Fractions. Unlike the associates of HP formed in water, molecules composing DF and OF are connected by covalent bonds. Analysis of the absorption spectra shows that the peak of the Cope band of solutions of DF and OF in D_2O (375 nm for DF and 365 nm for OF) corresponds to associated HP molecules. In detergent and alcoholic solutions this peak is shifted toward higher wavelengths and corresponds to HP solutions containing a mixture of monomeric and associated molecules (Fig. 2). These data indicate an essential change in the conformation of DF and OF in the media studied. Most probably DF and OF molecules in D_2O without detergent form a set of associates: unlike HP, moreover, the formation of intra- and intermolecular complexes can be suggested. In ethanol and in D_2O in the presence of detergent some of these complexes are evidently destroyed, forming structures similar in their spectral properties to monomeric HP.

TABLE 1. Relative Values of Quantum Yields of $^1O_2$ Generation by Porphyrins

Sensitizer	γ_Δ		
	D_2O	D_2O+2 % TX100	ethanol
TSPP	1,0	1,0	1,0
HPDHCl	0,35±0,05	1,05±0,07	1,10±0,07
HP	0,40±0,10	1,05±0,07	0,95±0,09
DF	0,15±0,05	0,75±0,05	0,85±0,04
OF	0,09±0,02	0,70±0,07	0,44±0,07

Legend. Asterisk indicates most probable absolute value of γ_Δ in all TSPP solutions used, namely 70 ± 5% [2, 10, 11]. (No asterisk given in Russian original — Publisher).

As Table 1 shows, the efficiency of $^1O_2$ generation in detergent-free aqueous solutions of DF and OF was significantly lower than in HP in the same media, OF being less active than DF. Thus the associated conformation of the polymeric HPD fraction possesses relatively low photodynamic activity. In alcoholic and micellar solutions of DF and OF a significant increase of γ_Δ was observed, although it did not reach the characteristic value for monomeric HP molecules. This result is evidently in agreement with the view that some intra- and intermolecular complexes may be destroyed through the action of an organic solvent or detergent.

Dimeric and oligomeric HP derivatives can thus generate $^1O_2$ in aqueous solutions. In this case, however, their activity is low and significantly less than the activity not only of monomeric, but also of associated HP molecules. The efficacy of $^1O_2$ formation rises sharply in organic media and micellar systems, simulating the lipid phase of biomembranes. Bearing in mind that polymeric components are more hydrophobic than HP and are located mainly in the lipid phase [5], it can be tentatively suggested that it is this effect which determines involvement of the dimeric and oligomeric components of Photofrin in the photodestruction of tumor cells and which plays a key role in the mechanism of photodynamic treatment of cancer.

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