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The incorporation of various porphyrins into blood cells measured *via* flow cytometry, absorption and emission spectroscopy[o]

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The incorporation of the five following porphyrins: *meso*-tetra(4-phenyl)porphyrin (TPP); *meso*-tetra(4-sulfonato-phenyl)porphyrin ($TPPS_4$); *meso*-tetra(4-naphthyl)porphyrin (TNP); tri-sulfo-tetra-phenyl porphyrin ($TPPS_3$) and tetra-sulfonato-naphthyl porphyrin ($TNPS_4$) into human blood cells was investigated using flow cytometry, and absorption and emission spectroscopy. The percentage of stained cells, measured in a fluorescence cytometer, provided information on the efficiency of incorporation of fluorescent dye molecules into different types of cells. The yield of the incorporation of a dye was dependent on the type of dye and the solvent used for cell incubation. The degree of dye aggregation and ionization varied with the incubation medium, but dye molecules incorporated into cells seemed to be restricted to those in the monomeric state, exhibiting similar fluorescence yield.

Of the three sulfonated porphyrins investigated only $TPPS_4$ was efficiently incorporated into leukocytes. In the incubation solvent, this dye was in monomeric and neutral form. $TPPS_3$ which was also in monomeric form, practically was not incorporated into cells.

TPP and TNP dissolved in 5% aqueous dimethyl sulfoxide were present mostly in aggregated forms but they penetrated the cells with a high efficiency.

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Abbreviations: DMSO, dimethyl sulfoxide; Mch, mean channel; TPP, *meso*-tetra(4-phenyl)porphyrin; TNP, *meso*-tetra(4-naphthyl)porphyrin; $TPPS_4$, *meso*-tetra(4-sulfonato-phenyl)porphyrin; $TPPS_3$, tri-sulfo-tetra-phenyl porphyrin; $TNPS_4$, tetra-sulfonato-naphthyl porphyrin; Solv. 1, 100% DMSO; Solv. 2, 5% DMSO in water.

The results obtained seem to indicate that porphyrins are promising candidates for application in photodynamic therapy.

Flow cytometry can be used to study incorporation of pigments into living cells [1-3]. Penetration of pigments into healthy and tumor cells is important for understanding of the mechanism of photodynamic effects [1-3].

In a previous paper [3] we have shown that, it is possible to evaluate the efficiency of incorporation of fluorescent dye molecules into leukocytes, by using a combination of flow cytometry and fluorescence yield measurements. The results were obtained for the human peripheral blood leukocytes incubated in the presence of stilbazolium merocyanine dyes. Merocyanines exhibited a toxic action on the blood cells even in darkness. Therefore, they are not suitable candidates for photodynamic therapy but can be useful as markers of leukemic cells because they are able to penetrate selectively into the membrane of resting and activated leukocytes [4]. Since porphyrins are claimed to be promising candidates for sensitizing the action of this therapy [5-10], therefore in this work we have evaluated the efficiency of the incorporation of various porphyrins into human peripheral blood cells.

MATERIALS AND METHODS

Synthesis of porphyrins

Meso-tetra(4-phenyl)porphyrin (TPP) was synthesized from pyrrole and benzaldehyde in propionic acid used as the reaction medium [11]. The dye was purified by reacting the crude product with 2,3-dichloro-5,6-dicyano quinone [11-13].

Meso-tetra(4-naphthyl)porphyrin (TNP) was synthesized from pyrrole and naphtaldehyde in propionic acid [14, 15]. TNP was purified by recrystallization from methanol/acetone (9:1, v/v) followed by the reverse-phase column chromatography on a silica 5 μm column.

For synthesis of *meso*-tetra(4-sulfonatophenyl)porphyrin ($TPPS_4$) [16], 308 mg TPP (0.5 mM) was dissolved in 15 ml fuming H_2SO_4 containing 30% free SO_3 (oleum) and heated at 85°C with stirring. The mixture was kept at this temperature for 6 h, then each fraction was poured onto crushed ice. The resulting dark green precipitate was filtered and washed with 1 M HCl (10 ml), redissolved in 1 M NaOH (20 ml) and filtered in order to remove insoluble in water impurities including non-sulfonated porphyrin. The filtrate was neutralized with 1 M HCl, analyzed by HPLC, concentrated in vacuum and stored at 0°C pending chromatographic purification. Because of strong technical difficulties in $TNPS_3$ purification we limited our experiments only to the tetra-sulfonatonaphthyl porphyrin ($TNPS_4$) form. The TNP (409 mg) was dissolved in 25 ml of oleum and was heated 6 h at 85°C with stirring. After 6 h, the solution obtained was poured onto crushed ice. After this, a similar purification process as used for $TPPS_4$ was followed.

$TPPS_4$, tri-sulfo-tetra-phenyl porphyrin ($TPPS_3$) and $TNPS_4$ with different degrees of sulfonation were separated on a 25 cm long reverse phase-column packed with ODS-2 spherisorb 5 μm, operated at 2 ml/min with a linear gradient (50 min) from 0% to 95% MeOH in 10 mM sodium phosphate buffer, pH 5. The porphyrins were detected by their absorbance at 412 nm [17]. Retention time varied from 20-25 min for $TPPS_4$ to 45 min for $TNPS_4$. The chemical structures of the porphyrins used are shown in Fig. 1 and in Table 1.

Staining of leukocyte cells

Whole heparinized human blood samples remaining after routine analysis were incubated with porphyrins. Porphyrin solution, 5 μl, (concentration 5×10^{-4} M) in water, in 100%

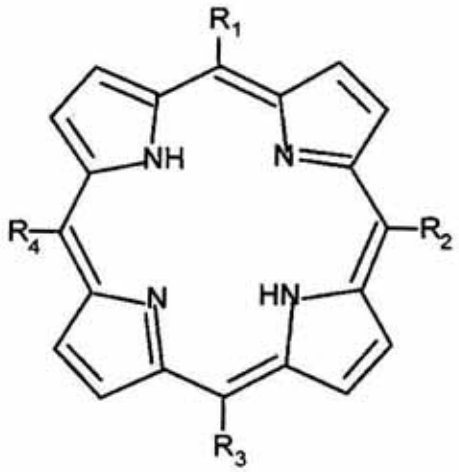

Figure 1. Scheme of the investigated porphyrins.

R_1-R_4 substituents are collected in Table 1.

DMSO (Solv. 1) or in 5% DMSO + 95% H_2O (Solv. 2) was added to 50 μl of blood. The last solution was prepared by first dissolving the dye in Solv. 1 and then adding water. The same volume of a solvent was added to control samples. Incubation was carried out at 25°C for 1 h, then the samples were treated with 1 ml of lysing reagent "ortho-mune" (Ortho Diagnostics, U.S.A.). The stained cells to be used in absorption and fluorescence measurements were washed after the incubation, and resuspended in unpigmented solvents.

Cytometric measurements

The Ortho Cytoabsolute Laser Flow Cytometry System, version 1.7 C (Johnson and Johnson, Raritan, NY, U.S.A.), equipped with an argon ion laser, was used. This instrument allows to measure the fluorescence emitted the cells in the three spectral regions: green, red and orange. A relationship between the intensity of forwardly and at right angle scattered light enable to establish the various types of cells. The fluorescence, excited at 488 nm, was measured through the orange band pass filter (563-607 nm) and through the red filter (λ > 620 nm) in the direction perpendicular to that of the exciting light and the cell stream. For every type of cells the fluorescence was analyzed separately. The percentage of stained cells and the average emission intensity of a given population of the cells was obtained from the Immunocounts program (Orto) using the gate analysis method [18].

The distribution of the fluorescent cell population between various cytometer channels was thus determined. The percentage of stained cells provided information on the efficiency of dye incorporation. The average intensity of fluorescence of the cells pigmented by a given dye was obtained from the so called „mean channel" (Mch), defined as $Mch = \Sigma N_i x_i / \Sigma N_i$, where N_i is the quantity of the cells registered in channel "i" and x_i – number of this channel. Number of channel x_i is the intensity of fluorescence due to the cells gathered in channel "i", it is expressed in arbitrary units the same for the whole set of measurements. Therefore, the fluorescence emitted by cells gathered in the "mean channel" can be used [3], as a measure of the mean fluorescence intensity of a given population of the stained cells. The mean fluorescence intensity

Table 1. Structures of the porphyrins used

Notation	R_1	R_2	R_3	R_4
TPP	C_6H_5	C_6H_5	C_6H_5	C_6H_5
TNP	$C_{10}H_7$	$C_{10}H_7$	$C_{10}H_7$	$C_{10}H_7$
$TPPS_3$	C_6H_5	C_6H_4-SO_3	C_6H_4-SO_3	C_6H_4-SO_3
$TPPS_4$	C_6H_4-SO_3	C_6H_4-SO_3	C_6H_4-SO_3	C_6H_4-SO_3
$TNPS_4$	$C_{10}H_7$-SO_3	$C_{10}H_7$-SO_3	$C_{10}H_7$-SO_3	$C_{10}H_7$-SO_3

TPP, 5,10,15,20-tetra-phenyl-porphyrin; TNP, 5,10,15,20-tetra-naphthyl-porphyrin; $TPPS_3$, 5,10,15-tri-sulfo-tetraphenyl-porphyrin; $TPPS_4$, 5,10,15,20-tetra-kis-sulfo-phenyl-porphyrin; $TNPS_4$, 5,10,15,20-tetra-kis-sulfo-naphthyl-porphyrin.

depends on the number of incorporated dye molecules as well as on the fluorescence yield of a dye in the cell.

The measured fluorescence intensities were proportional to the light emission over the entire spectral region used.

The fluorescence of lymphocytes and granulocytes was analyzed separately. The number of stained cells of a given type was obtained from the percentage of gated cells exhibiting the observed fluorescence. These numbers provide information on the extent of incorporation of a dye into the cells, assuming that only fluorescent forms of a given dye were incorporated into the cells. The absorption of stained cells in the wavelength region used for the excitation in the cytometer was low but sufficient to record measurable fluorescence of the cells. In the case of strongly different average numbers of dye molecules incorporated per one stained fluorescent cell, the average fluorescence intensities (Mch values) should be very different on the assumption that the incorporated dyes exhibit comparable fluorescence yields. It is possible that the interactions between pigment and the material of the cell membrane can cause disaggregation of the dye, so that predominantly monomeric forms became incorporated. This supposition was verified by comparison of the ratios of the mean channel values of fluorescence cytometry and relative fluorescence yields of dyes in the cells.

Absorption and fluorescence spectra

In order to evaluate the state of aggregation and ionization of the dye incorporated into cells, the absorption and fluorescence spectra of dyes in incubation solvents and in the cells were compared. On the basis of these results the relative yields of fluorescence of various dyes in different conditions were established. The various dyes were aggregated or/and ionized to different degree in incubation solvents therefore they exhibited different absorption spectra [9, 17]. The absorption spectra were recorded using a Specord M40 spectrometer (Carl Zeiss Jena, Germany), while the fluorescence spectra by means of a spectrofluorimeter constructed in our laboratory and described previously [19].

RESULTS AND DISCUSSION

Absorption and fluorescence spectra of dyes in incubation solvents

The absorption spectra of the investigated dyes (Figs. 2 and 3) were obtained in solvents used for cell incubation: Solv. 1 and in Solv. 2, respectively. Similar solvents were used to apply various porphyrins to tumors and cells [6]. Thus application of these solvents allowed to compare our results with available literature data [6, 20, 21]. In Solv. 1 all porphyrins studied had similar spectra, with their Soret bands located in the region from 418 nm to 420 nm. These spectra are similar to the spectra of other porphyrins in typical organic solvents [10], but exhibit a shift of the Soret band from 412–413 nm to 418–420 nm, probably due to

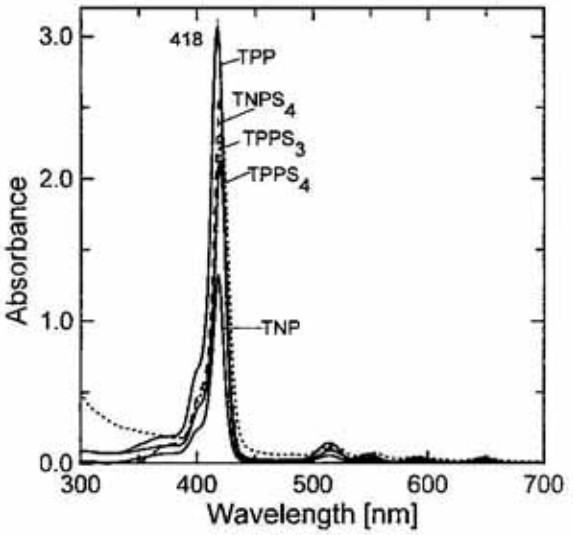

Figure 2. Absorption spectra of investigated porphyrins in 100% DMSO.

Concentrations of dyes from 2.5×10^{-4} M to 5×10^{-4} M.

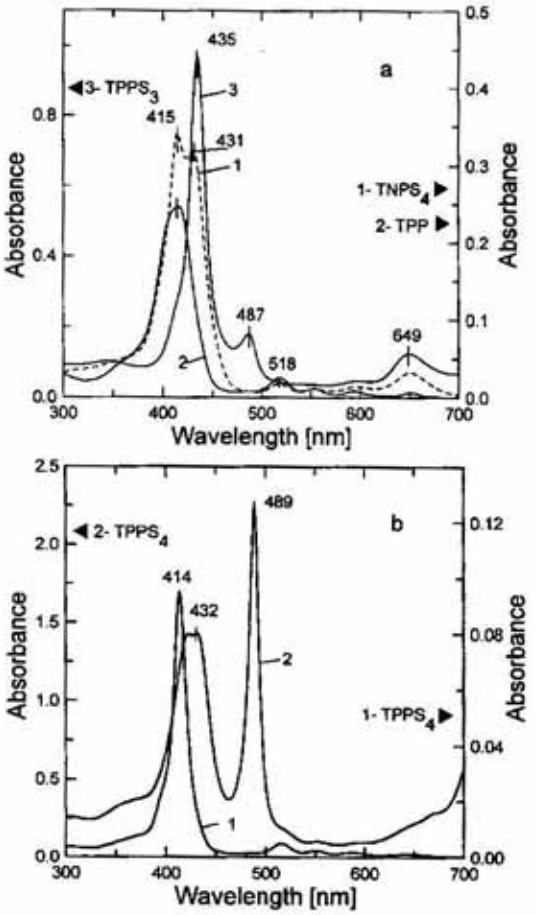

Figure 3. Absorption spectra of porphyrins in 5% aqueous DMSO.

a) TPP, 5×10^{-4} M; $TPPS_3$, 5×10^{-4} M; $TNPS_4$, 2.5×10^{-5} M; b) $TPPS_4$ right scale (2.5×10^{-5} M) just after water addition; left scale (5×10^{-4} M) one hour after water addition.

an interaction of the dyes with the solvent used (Figs. 3 and 4). The various heights of bands are due to different dye concentrations. The dyes in Solv. 1 differ in solubility but all the spectra exhibit patterns characteristic of monomeric forms of the dyes and have similar molar absorption coefficients. Addition of water to DMSO (Solv. 2) caused strong changes in the spectral shapes and intensities (Fig. 3). In the case of TPP (Fig. 3a), these changes resulting from water addition were characteristic of the aggregation of the dye. The main absorption band was located at about 416 nm and broader than that of the monomeric form. The absorption ratios of the four weaker bands, characteristic of porphyrins, were also changed. The Q_y band was shifted from 515 nm to 518 nm. The molar absorption coefficient of the main band was diminished. This indicates formation of large oligomers, because in the case of dimerization this coefficient was found to increase [9]. Similar changes were observed for TNP in Solv. 2 (Fig. 3a). As it follows from a comparison of the spectra shown in Fig. 3a, b and Fig. 4 with those reported in the literature [22, 23], the spectra of the sulfonated compounds $TPPS_4$, $TNPS_4$ and $TPPS_3$ in Solv. 2 were very similar to those of a bicationic form. The absorption spectra of $TPPS_4$ at various pH values [6, 11, 22] suggest that, with a decrease in pH, the ring nitrogens of the dye are protonated with formation of an almost symmetrical non planar dicationic form. As a result, the Q_x band becomes degenerated and instead of two Q_x bands at 580 nm and 634 nm only one strong component at 645 nm appears. The Soret band is shifted from 413 nm to 435 nm and the intensity of the Q_y bands at 515 nm and 552 nm decreased. A similar spectral pattern for $TPPS_4$ in Solv. 2 was also observed by us (Figs. 3 and 4d); the spectrum of $TNPS_4$, represent a superposition of the spectra of the monomeric neutral and bicationic forms. In Solv. 2, the absorption of $TPPS_4$ (Fig. 3b) changed with time. Initially, after the addition of water to the concentrated DMSO solution of this dye, the spectrum characteristic of monomeric form was observed (Fig. 3b, right scale), but after a few hours the dye becomes partially protonated to the bicationic form, as indicated by a strong increase in the intensity of the absorption band at 489 nm (Fig. 3b, left scale). Similar changes were observed for this dye at the strongly acidic pH range of 1–2 [22, 23].

The fluorescence spectra of the same dyes were excited at 488 nm, *viz.* at the same wavelength as used in the cytometric measurements. In Solv. 1 the shapes of the fluorescence spectra of all dyes are very similar (Fig.

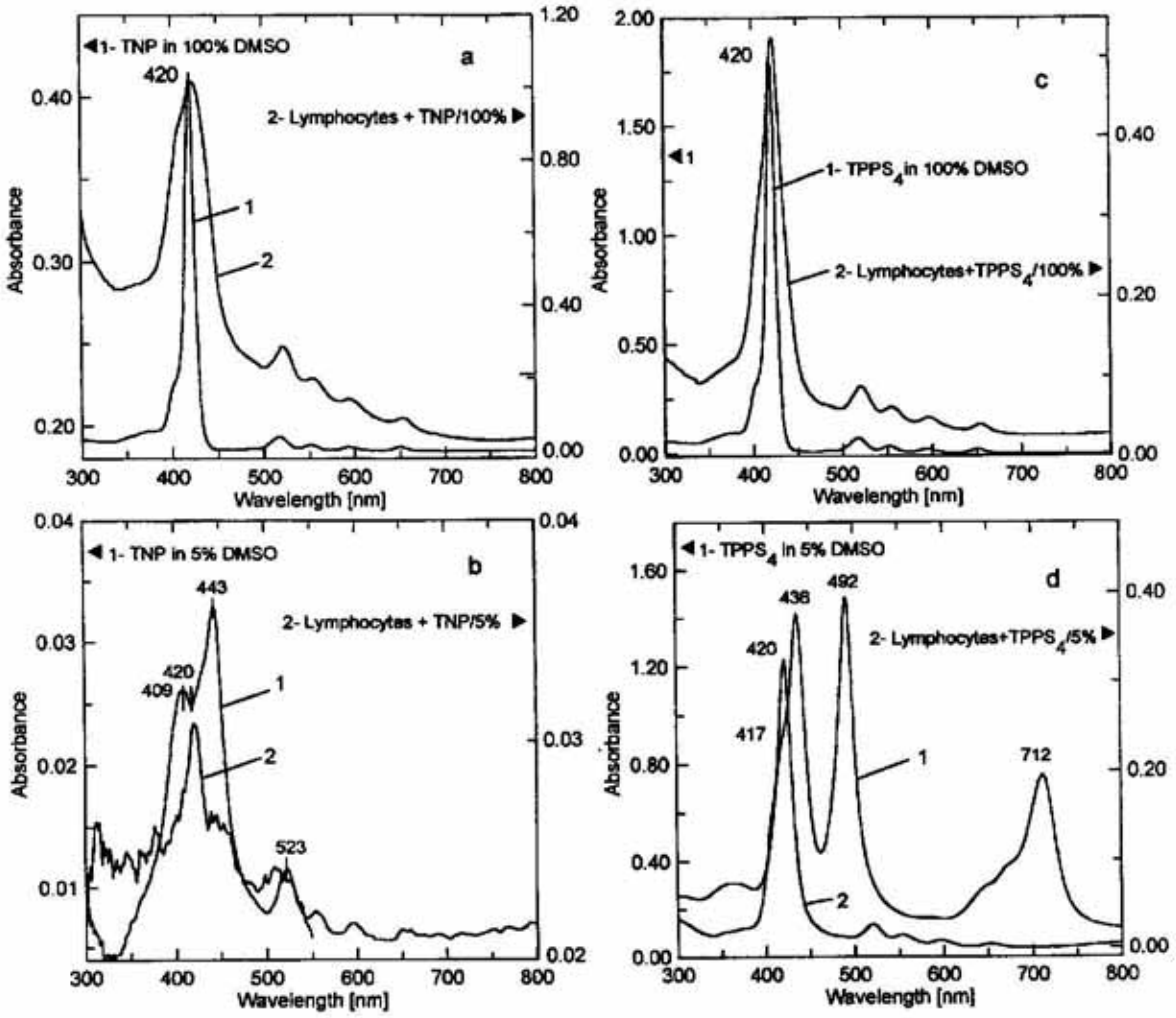

Figure 4. The absorption spectra of porphyrins in incubation solvents and incorporated from these solvents into the cells.

a) TNP in 100% DMSO, b) TPN in 5% aqueous DMSO, c) $TPPS_4$ in 100% DMSO, d) $TPPS_4$ in 5% aqueous DMSO. Curves 1, in solvent; 2, in the lymphocytes.

5), with maxima located at 650–652 nm and 714–717 nm, characteristic of monomeric forms of porphyrins.

The fluorescence intensities of TPP and TNP in Solv. 2 (Figs. 5b and 6b,c) were several times lower than those found in Solv. 1. This observation showed again that these dyes in Solv. 2 were present in different forms than in Solv. 1. The shapes of long wavelength emission bands and the ratio of the intensities of the two bands were changed. The shift of the main fluorescence band as well as the occurrence of an additional long wavelength shoulder at 750 nm in the case of TPP, suggests that some dye aggregates or its associates with solvent molecules could be slightly fluorescent. A shift of the main fluorescence band of $TPPS_3$ to 673 nm in Solv. 2, in which this dye is in bicationic form (Fig. 5b), suggests that the bication emits in this region. The emission of $TPPS_4$ in Solv. 2 was found to depend strongly on the excitation wavelength: at 412 nm, two fluorescence maxima at 672 nm and 749 nm were observed, whereas at 488 nm a predominant maximum at 724 nm was seen (not shown). The latter should be related

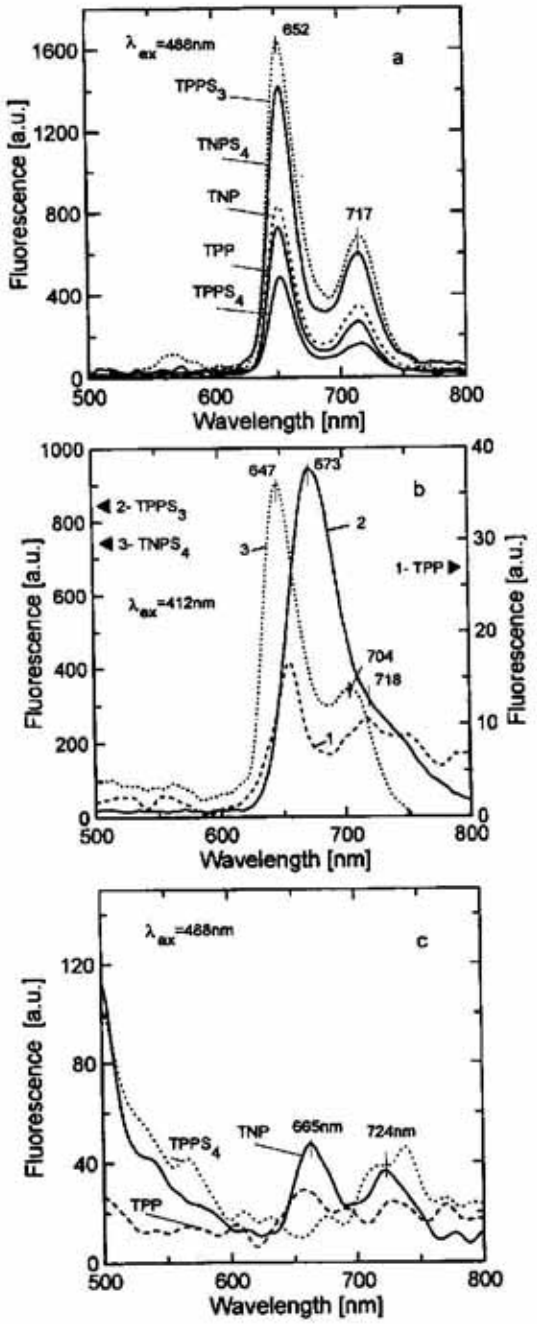

Figure 5. Fluorescence spectra of porphyrins.

a) in DMSO (5×10^{-4} M), b) in 5% aqueous DMSO, 5×10^{-4} M, λ_{exc} = 412 nm, c) λ_{exc} = 488 nm, 5% aqueous DMSO; a.u., arbitrary units.

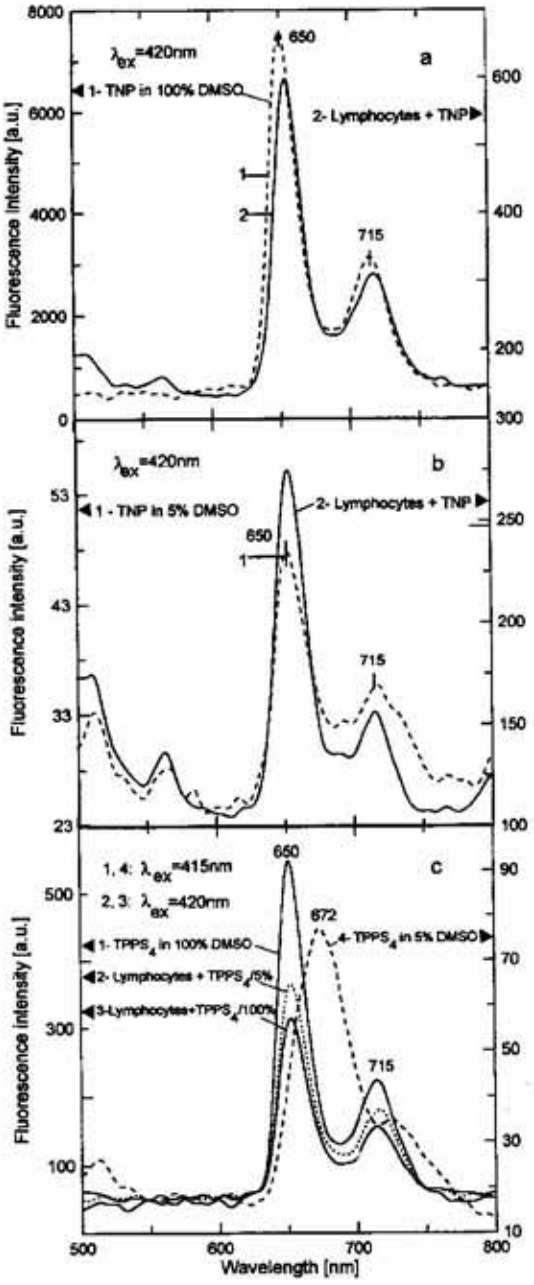

Figure 6. Fluorescence spectra of porphyrins in incubation solvents and in the lymphocytes.

a), b) TNP. Curves 1, in solvent; curves 2, in the cells. c) $TPPS_4$: 1, in 100% DMSO; 2, in cells incubated in 5% aqueous DMSO; 3, in cells incubated in 100% DMSO; 4, in 5% aqueous DMSO. Wavelengths of excitation are given in the Figure.

to the form exhibiting strong absorption at 489 nm. As it follows from the absorption spectra shown in Fig. 3b, $TPPS_4$ in Solv. 2 is a mixture of several forms. The same conclusion can be drawn from its fluorescence spectra. In Solv. 1, $TNPS_4$ exhibits a strong fluorescence, characteristic of monomeric porphyrins. According to the absorption spectrum, this dye in Solv. 2 occurs as a mixture of

monomeric neutral and bicationic forms (Fig. 3a). It seems that in this case bication exhibits low intensity fluorescence. Inspection of Figs. 5 and 6 shows that the fluorescence spectra of the investigated porphyrins depend strongly on the presence of added water, which causes aggregation and/or formation of bicationic forms.

Spectral properties and fluorescence quantum yield of dyes in leukocytes

The quantum yield of fluorescence of most porphyrins incorporated into the cells is low, usually ϕ_f about 0.01 [6, 9, 10]. The dyes investigated by us also exhibited low ϕ_f when incorporated into cells. Therefore it was possible to establish ϕ_f only for the dyes able to become incorporated rather efficiently into cells. The differences found in aggregation or ionization properties of the dyes in the solvents and in the cells, caused the changes in their respective fluorescence yields. It will be exemplified by the data for two dyes: TNP and $TPPS_4$.

The absorption and the fluorescence spectra of these two dyes were recorded in both incubation solvents and in the cells (Figs. 4 and 6). The main absorption maximum of TNP in Solv. 1 was located at 420 nm, whereas in the cells the maximum was broader and located at 421 nm. The shift and a change in the band shape were possibly due to an interaction between the dye and cell material. Both maxima are belived to be due to the monomeric form of the dye [9, 10]. The fluorescence spectra (Fig. 6) of the two samples are almost identical, indicating that the same monomeric fluorescent form of the dye was present both in Solv. 1 and in the cells. The absorption of TNP in Solv. 2 indicates that in this solvent the dye was aggregated in a high degree; the three observed maxima at 409 nm, 443 nm and 523 nm are characteristic of TNP aggregates [9–11]. In lymphocytes, the absorption maximum at 420 nm characteristic of TNP monomers predominates.

The spectrum of $TPPS_4$ in Solv. 1 exhibits also monomeric band at 420 nm which in cells is broader and a little shifted towards longer wavelengths. The spectrum of $TPPS_4$ in Solv. 2, exhibits very well resolved bication maxima at 436 nm and 492 nm, with a low intensity shoulders at 417 nm and at 712 nm [22, 11], whereas in the cells the monomer absorption at 420 nm predominates. The apparent pH of Solv. 1 (pure DMSO) was 8.6, whereas that of Solv. 2 (5% aqueous DMSO) it became 4.0. Such a change in pH of this dye in the binary Solv. 2 was also reported by Ion [23]. At low DMSO content, as in Solv. 2, the solution was acidic, in agreement with the presence of the bicationic form. The solution of TNP in the same solvent did not exhibit any pH change. The fluorescence spectra of the cells incubated in both solvents with $TPPS_4$ are similar and identical to those of TNP under the same condition (Fig. 6). In Solv. 2 the $TPPS_4$ spectrum was much broader than in Solv. 1 or in the cells. In Solv. 2 $TPPS_4$ is in the bicationic form [20, 22]. The fluorescence spectrum of $TPPS_4$ in Solv. 2 depends on the wavelength of excitation, but in the cells incubated in this solvent only emission of neutral monomers was seen at all wavelengths of excitation. The broad band due to aggregates or/and bications emission was of much lower intensity than the fluorescence band of the same sample excited with the spectral region of the monomer absorption. As follows from the absorption spectra (Fig. 2), the ionized or aggregated forms of the dyes are present in the sample at a much higher concentration than monomers. Low intensity emission of aggregated and ionic forms is due to much lower ϕ_f of these forms than that of the monomer fluorescence. The results presented in Figs. 4 and 6 show that, independently of the state of a dye aggregation or ionization in incubation solvents, the dye molecules incorporated into cells are predominantly in the neutral monomeric form.

The relative fluorescence yields of various dyes in the same cell type was established on

the basis of the measured absorption and fluorescence spectra. The ratio of ϕ_f for $TPPS_4$ and TNP (both incubated in the Solv. 2) was equal to 1.1 ± 0.2. It was also found that ϕ_f for both these dyes was much higher in the cells than in Solv. 2, what can be explained by a much lower ϕ_f of dye aggregates or bications in aqueous solvents than the yield of monomeric forms incorporated into cells.

Flow cytometer results

An exemplary cytogram of a blood sample investigated, showing the dependence of the forward light scattering on the right angle scattering is given in Fig. 7. It allows to distinguish the regions A, B and C corresponding to Scatterd light from lymphocytes, granulocytes and monocytes, respectively. The fluorescence of cells belonging to types A and B was analyzed separately. The fluorescence of monocytes seen in Fig. 7 (region C) was not further analyzed.

The representative fluorescence histograms, showing the distribution of granulocytes emitting in the red region offer incubation with TNP in Solv. 1 and in Solv. 2 are shown in Fig. 8a and 8b, respectively. Very low intensity of emission (below 10 arbitrary units of fluorescence intensity) is due to the inherent fluorescence of the sample, observed even for cells not incubated with a dye. The distribution of fluorescent cells in regions of stronger emission (Fig. 8) provided information about stained cells. The percentage of stained cells gated and mean fluorescence of the stained cells after incubation with the various dyes obtained from the analysis of cytometric results, such as in Fig. 8, are collected in Table 2. The

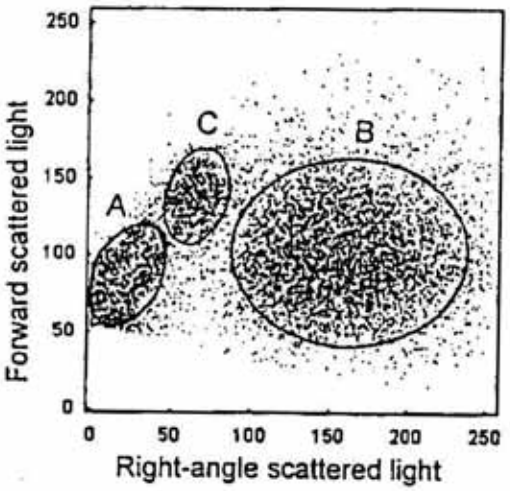

Figure 7. The dependence of forward light scattering on the right-angle scattering for one of the investigated blood samples (after the addition of lysing agent).

Region of: A, lymphocytes; B, granulocytes; C, monocytes.

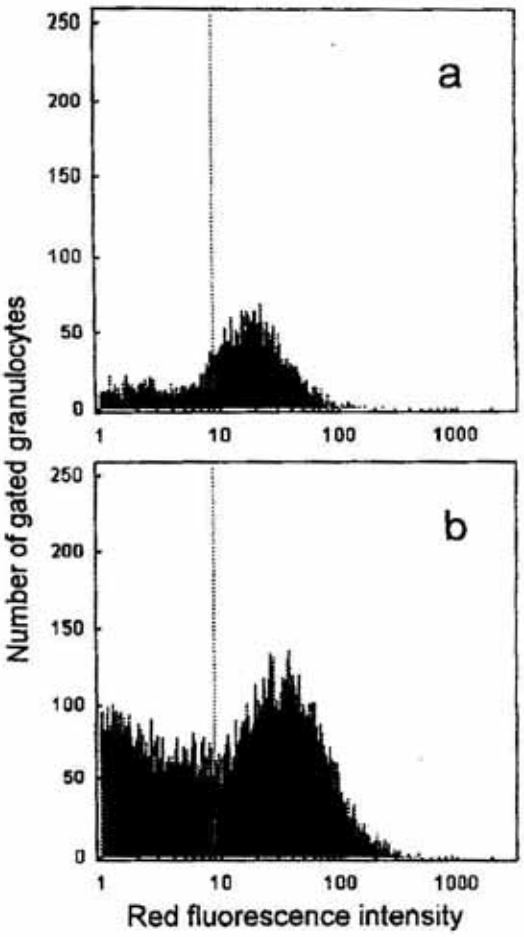

Figure 8. Examples of the fluorescence histogram (distribution of red fluorescing leukocytes stained by TNP).

a) Granulocytes incubated in 100% DMSO; b) granulocytes incubated in 5% aqueous DMSO. Filter – red ($\lambda >>$ 620 nm). The fluorescence of stained cells could be observed for fluorescence values higher than 10 a.u.

Table 2. Incorporation of the dyes into cells

Dye	Cells*	Solvent**	Stained cells (%)	Mean intensity of fluorescence	State of dye***
TPP	L	1	1.6	87.0	M
TPP	G	1	12.0	120.0	M
TPP	L	2	4.5	82.3	A
TPP	G	2	63.8	113.1	A
TPN	L	1	4.7	86.3	M
TPN	G	1	36.1	123.0	M
TPN	L	2	4.5	69.0	A
TPN	G	2	63.8	92.0	A
$TPPS_3$	L	1	~0.0	114.0	M
$TPPS_3$	G	1	~0.0	75.0	M
$TPPS_3$	L	2	0.1	122.1	B
$TPPS_3$	G	2	0.2	93.1	B
$TPPS_4$	L	1	44.0	89.7	M
$TPPS_4$	G	1	67.0	121.0	M
$TPPS_4$	L	2	0.0	91.0	B+D
$TPPS_4$	G	2	0.1	77.7	B+D
$TNPS_4$	L	1	~0.1	87.7	M
$TNPS_4$	G	1	~2.6	96.3	M
$TNPS_4$	L	2	~0.0	~0.0	M+B
$TNPS_4$	G	2	~0.1	72.0	M+B

*L, lymphocytes and G, granulocytes; **1,100% DMSO, and 2.5% DMSO in water; *** In solvent: M, monomer; A, aggregate; B, bication; D, denatured form.

percentage of the fluorescent cells gated for a non-incubated sample was zero within the accuracy limit. Similar results were obtained for the incubation of leukocytes in water solutions of dyes. The percentage of gated fluorescent cells was always higher for granulocytes than for lymphocytes. Also Mch values were found higher for granulocytes than for lymphocytes (Table 2). This indicates more efficient incorporation of dyes into the first rather than into the second type of cells. The mean channel fluorescence intensity for the cells incubated with either solvent for various types of dyes and the same group of leukocytes, is similar. This observation suggests that similarly fluorescent forms of dyes are gathered in the cells irrespective of which of the two solvents was used for incubation. The percentage of gated fluorescent molecules depends strongly on the addition of water to the incubation solvent (Table 2). The results obtained show thus that the uptake of the dye molecules depends on aggregation of the dye in the incubation solvent.

In the case of TPP the percentage of stained fluorescent cells in 5% aqueous DMSO was much higher than in DMSO alone. As it follows from absorption and emission spectra of this dye, it remains in a highly aggregated state in the aqueous solvent. But the mean intensities of fluorescence from the cells are very similar to these observed for other dyes (Table 2), which suggests that dye, contained must probably in a membranous structure becomes disaggregated due to its interaction with lipids, and therefore exhibits a similar mean intensity of fluorescence as does its monomeric form incorporated from Solv. 1.

The process of disaggregation of dyes in the membrane has been confirmed by absorption and fluorescence spectra of stained leukocytes (Figs. 4 and 6) and is supported by the results obtained for model systems [21, 22, 24, 25] showing that aggregation depends on the lipid–chromophore concentration ratio. In all cases, penetration of the dye molecules into granulocytes was more efficient than into lymphocytes. Of the sulfonated compounds only $TPPS_4$ exhibited efficient penetration into cells, but only in the Solv. 1 in which this dye does not become aggregated. The other sulfonated porphyrins ($TPPS_3$ and $TNPS_4$) exhibited very low percentages of gated leukocytes. It was reported that with an increase in the hydrophobicity of porphyrin derivatives [26] and for the less sulfonated derivatives of TPP [7] penetration into leukocytes was enhanced. The increased photoactivity of the less sulfonated derivatives was attributed to their higher hydrophobicity and better cell-penetrating properties [7]. The sulfonated porphyrins, such as *meso*-tetra-phenyl-porphyrin with n-peripheral sulfonate substituents at *para* positions (n = 1, 2, 3 or 4), considerably change their properties from lipophilic (n = 0 or n = 1) to hydrophilic (n = 3 or n = 4) [9]. This shows that porphyrins, depending on the side-chain substitution on the tetrapyrrol ring, can become very lipophilic. This explains the lower inefficient penetration of leukocytes by $TPPS_3$ and $TNPS_4$ (Table 2). However, in the case of $TPPS_4$, hydrophobicity of this compound does not seem to be the most important factor influencing penetration [27].

Photochemical activity of porphyrins is unaffected by the number of sulfonate substituents, as long as the dye is in its monomeric form [28, 29]. Therefore, in the case of disaggregation of a dye in the membrane, all the activity of all the investigated dyes can be similar. However, the dyes differ strongly in the efficiency of penetration because of their being in various form in the incubation medium (Table 2). It seems that all the investigated porphyrins are introduced into cells in the monomeric form. Although some dimeric porphyrin derivatives appear to display an optimal tumor-localizing activity [30, 31], aggregation decreases the probability of porphyrin photoexcitation [22, 32, 33] and hence also of singlet oxygen formation, if face-to-face clusters are formed. On the other hand, end-to-end planar configurations exhibit emission properties comparable to those of the monomer [22, 33]. At the present state of our investigation we are not able to reach a conclusion concerning the possible type of the occurring aggregates.

It has been reported that protonation of the inner nitrogen atoms drastically reduces the hydrophobicity of the porphyrin core and thus prevents incorporation [9]. Our results and some literature data [33] show that this is not a general rule.

Photostability of dyes

The porphyrins used in photodynamic therapy can be photo-oxidized [17, 34]. Therefore the stability of dyes has to be established in incubation solvent as well as in the cells. The degradation efficiency depends on the type of *meso*-substituents and on the type of solvent used [33]. The conditions in which the photodegradation of several types of porphyrins occurs were reported by Ion [17].

The changes in fluorescence of leukocytes following their illumination are complex. For example, when granulocytes were incubated with TNP solution in Solv. 1 before illumination, 46% of the cells became stained; after 1 s of illumination with Hg lamp (intensity of light 9500 lux) there was only 9.4% of stained granulocytes gated, but after 10 s of illumination, the percentage of stained cells was increased again to 36.1% (not shown).

For the photodynamic therapy of cancer it is important to know the contribution of each reactive oxygen species (singlet, superoxide, hy-

droxyl, etc.) to the photo-oxidative reaction of porphyrins. However, on the basis of the results presented we were not able to establish what are the photoreaction products.

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