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QUARTERLY

# The interactions of phthalocyanines with stimulated and resting human peripheral blood mononuclear cells✪

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**The interactions of two metal-free phthalocyanines [($H_2Pc$) and Solar Pc (with four peripherical groups: $SO_2N(CH_2CH_2OH)_2$)] and of one metal substituted dye (CoPc) with resting and stimulated human peripheral blood mononuclear cells (PBMC) were compared.**

**The absorption, fluorescence, photoacoustic and EPR spectra of both resting cells and cells stimulated by phytohaemagglutinin, incubated in dimethyl sulfoxide (DMSO) with very low or 95% water content and with or without dye addition, were measured. The fate of the light absorbed by the samples was investigated. It is known that singlet oxygen production is crucial for photodynamic action of dyes. Thermal deactivation and luminescence emission compete with this process, so investigation of these alternative paths of sensitizer deactivation provides information about photodynamic action. The incorporation of the investigated dyes into cells and the perturbation of the cell structure caused by the dyes, the incubation solvent and the activator were investigated by comparing the spectral properties of PBMC before and after stimulation and incubation. Incubation of the cells for 1 h in a solution of Solar Pc in 99.5% aqueous DMSO, resulted in an efficient dye incorporation which was highly selective. Solar Pc being introduced much more efficiently into stimulated cells than into resting cells.**

Phthalocyanines (Pcs) are dyes which show a long wavelength absorption band in the 675–700 nm region, in which cells are relatively transparent [1–3]. They can be chelated by various metals elements and several substituents can be placed at their periphery. Metal chelation and substitu-

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**Abbreviations:** DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; PAS, photoacoustic spectra, photoacoustic signal; PBMC, peripheral blood mononuclear cells; Pc, phthalocyanine; PDT, photodynamic therapy; PHA, phytohemagglutinin; PI, propium iodide; PVA, polyvinyl alcohol; TD, thermal deactivation.

tion strongly influence the solubility and toxicity of the dyes, as well as the efficiency of incorporation of the molecules into cells. Molecules with long living triplet state cause efficient photosensitization. Pcs with diamagnetic metals such as Co seem to be better suited for photodynamic therapy (PDT) than those with paramagnetic metals (such as Cu or Fe) because the latter photoinactivate the dyes by shortening the lifetime of the triplet state. In reality the situation is more complex, because even in the metal-free $H_2Pc$ free radicals can be formed due to breaking of $\pi$-bond in the phthalocyanine matrix [4, 5]. As concerns CoPc, the unpaired 3d electrons associated with the Co atom could interact with the ligating nitrogens of Pc [5] giving rise to the EPR signal. Therefore experimental EPR data are needed to determine the photochemical effect of Pc on the investigated cells. It has been shown [1–3] that sensitizers applied in PDT should exhibit in addition to photoreactivity a low toxicity in darkness and their uptake into normal and neoplastic (malignant) cells should be highly selective.

Metal-free Pcs are poor sensitizers and do not kill the cell [1]. Sulfonated Pc dyes are usually good sensitizers [1]. We have therefore, compared the fate of the absorbed light in the metal-free $H_2Pc$ with that in Solar Pc in which the $H_2Pc$ is substituted by four peripherical groups [$SO_2N$ -($CH_2$-$CH_2$-OH)$_2$], as well as in the phthalocyanine substituted with Co (CoPc). All these dyes were introduced into resting and stimulated leukocytes. We are predominantly interested in deexcitation of illuminated dyes undergoing deactivation by various paths. Since such deactivations compete with the photochemical reaction caused by the sensitizing dye, we have measured the fluorescence and thermal deactivation of the samples. The EPR spectra of the investigated Pcs in DMSO which was the solvent used for incubation were also measured. It is known [6] that various activators, which are supposed to change normal (resting) peripheral blood mononuclear cells (PBMC) into neoplastic (malignant) cells, can differ in their influence on the structure of the cells. Therefore all the experiments were carried out with the same activator, phytohaemagglutinin (PHA).

## MATERIALS AND METHODS

***Dyes synthesis***. The structures of the investigated phthalocyanines are shown in Fig. 1.

The free-base phthalocyanine ($H_2Pc$) (Fig. 1a) was prepared and purified according to Moses & Thomas [7]. The cobalt phthalocyanine (CoPc)

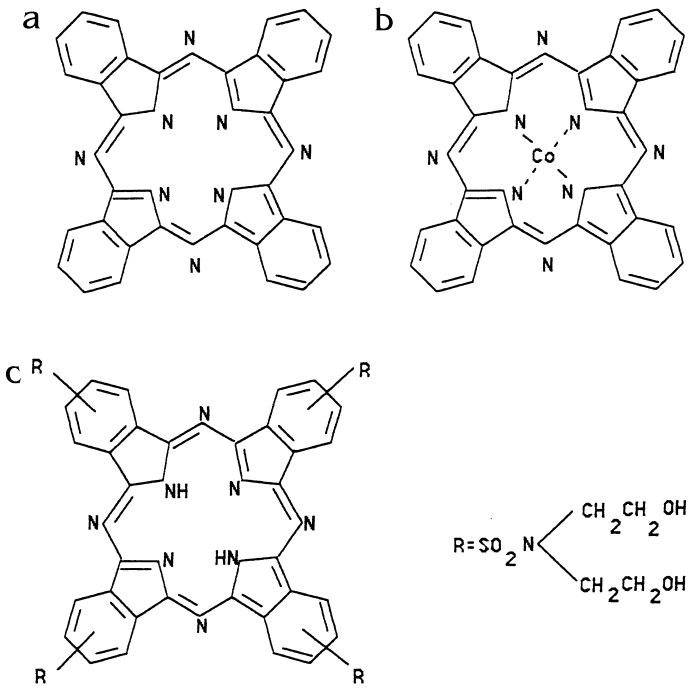


**Figure 1. Structures of the investigated phthalocyanines.**

a, $H_2Pc$; b, CoPc; c, Solar Pc.

(Fig. 1b) was prepared as described in the literature [8–10]. The Solar phthalocyanine (Fig. 1c) was prepared by a two stage procedure starting with $H_2Pc\ (SO_3H)_4$ and di-ethanolamine as described by Ion [11].

***Incubation of the cells.*** The stimulation and staining of PBMC were carried out as described previously [2, 3].

Whole heparinized human blood samples remaining after routine analysis were used in experiments. The purity and number of mononuclear cells (monocytes and lymphocytes) were established by flow cytometry. One part of the sample was stimulated using PHA (phytohemagglutinin HA17, Wellcome, England) at a concentration of 10 $\mu$g/ml during 1 h at 37°C. The second part of the sample was not stimulated. Both the stimulated and resting cells were divided into stained samples and control samples. Dye Pc solution at a concentration of $10^{-2}$ M or $10^{-4}$ M in 99.5% DMSO (denoted in the text DMSO) or in 5% aqueous DMSO was added to a suspension of mononuclear blood cells containing $3.6 \times 10^8$ cells. The dye solution, (1 ml), was added to a such a volume of cell suspension that 1 $\mu$l of final suspension contains $10^6$ cells. The same volume of the solvent without dye was added to control samples. Incubation was carried out at 37°C for 15 min or 1 h. The stained cells were washed after the incubation, and then resuspended in pure solvents.

The analyzed cell populations contained about 95% of mononuclear cells, mainly lymphocytes (70–75%). In the cytometric analysis the purity of lymphocyte gate was over 97%, as controlled by CD14/CD45 monoclonal antibodies staining.

In control experiments we checked the influence of various reagents on cell viability, using propidium iodide (PI) staining for estimating the percentage on the dead cells. The population of viable cells after Gradisol isolation was about 90% and addition of DMSO or dye dissolved in DMSO reduced it to 80%. In other experiments, to avoid interference of PI fluorescence with fluorescence of the investigated dye, the gate analysis based only on changes of light scattering on dead cells was used, but the results were comparable with those of PI staining.

Part of the dye was embedded into a polymer polyvinyl alcohol (PVA) film as described previously [12]. Such samples are photochemically very stable and can therefore be used for measurements requiring strong illumination for a long time (e.g., when measuring PAS at different frequencies of light modulation). The results for the DMSO solutions and the PVA films were compared.

***Cytometric measurements.*** An Ortho Cytoron Absolute Laser Flow Cytometer, version 1.7C (Johnson and Johnson, Raritan, NY, U.S.A.) equipped with an argon ion laser, was used. The fluorescence excited at 488 nm was measured through a green filter (514–545 nm). The fluorescence of lymphocytes and monocytes was analyzed separately. The average fluorescence intensity emitted by stained and unstained cells was measured. It was obtained from the so called "mean channel" (Mch) value defined as $Mch = \Sigma N_i x_i / \Sigma N_i$, where $N_i$ is the number of cells registered in channel "i" and $x_i$ the channel number, i.e. the fluorescence emitted by the cells gathered in channel "i". This value is expressed in arbitrary units adopted for whole set of measurements. The fluorescence gathered in the "mean channel" can be used [3, 13] as a measure of the mean fluorescence intensity of a given population of stained cells. This intensity depends both on the number of incorporated dye molecules and on the fluorescence yield of the dye in the cells, the latter often much lower than that in solution [14]. Also the degree of dye aggregation in the cells and in solution is usually different [2, 12]. Approximate information about the incorporation of various dyes into cells can be obtained from the mean channel fluorescence and the fluorescence yield of dyes in incubation solvent. This relies on the crude supposition that the decreases of the fluorescence yield due to dye incorporation for a set of similar dyes are not dramatically different.

***Absorption and fluorescence spectra.*** The absorption spectra were recorded using a Specord M40 spectrometer (Carl Zeiss, Jena, Germany), and the fluorescence spectra were obtained using a spectrofluorimeter constructed in our laboratory [15].

***Photoacoustic spectra.*** The photoacoustic spectra were taken using a single beam spectrometer built in our laboratory, based on the apparatus made in Trois-Rivieres (Canada) [16]. In some sets of the measurements it was equipped with a different type of photoacoustic cell (Model 300, MTEC Photoacoustic, Inc. U.S.A.). The sample placed in a photoacoustic cell was illuminated by modulated light. The heat generated in a sample reached the surface between sample and working gas. The thin layer of warmed gas works as a piston on the rest of the gas and generates the acoustic wave acting on a microphone. The acoustic signal is preamplified and sent to phase lock-in-amplifier. The reference signal for the amplifier is provided by a chopper. The phase shift between incident modulated light and periodic acoustic signal is set so as to obtain the maximum amplifier output signal. The signal is computer processed. The obtained photoacoustic spectrum of the sample is corrected with use of the spectrum of the carbon black in order to eliminate the spectral distribution of lamp. The steady-state photoacoustic spectra measured at various frequencies of light modulation enable to establish the spectral region to which slow components of thermal deactivation contribute [17].

***EPR spectra.*** The EPR spectra were recorded using a Radiopan SE/x-2540 spectrometer with an RCX-660a resonator operating in $TM_{110}$ mode. For room temperature experiments samples were placed in a flat EPR cell and centered at the position of the electric field minimum inside the microwave resonator. Some of the EPR spectra were measured at 77 K.

## RESULTS AND DISCUSSION

### Spectral properties of phthalocyanines in incubation solvents (in DMSO or in 5% aqueous DMSO)

Figures 2a–c show the absorption spectra of the investigated Pcs (presented in Fig. 1) dissolved in DMSO at an intended concentration of $10^{-2}$ M. Figure 2d shows the absorption of the Solar Pc dissolved in 5% aqueous DMSO. All the dyes exhibit absorption in 550–700 nm region and in the short wavelength region. It seems that CoPc was not wholly dissolved, because the spectrum exhibits a high level scattered light, due to microcrystals observed under a microscope. The most efficiently dissolved was Solar Pc which exhibited high absorption and low scattering in either solvents. However the ratio of the short wavelength maximum to the long wavelength maximum increased with the water addition, which suggests increased dye aggregation.

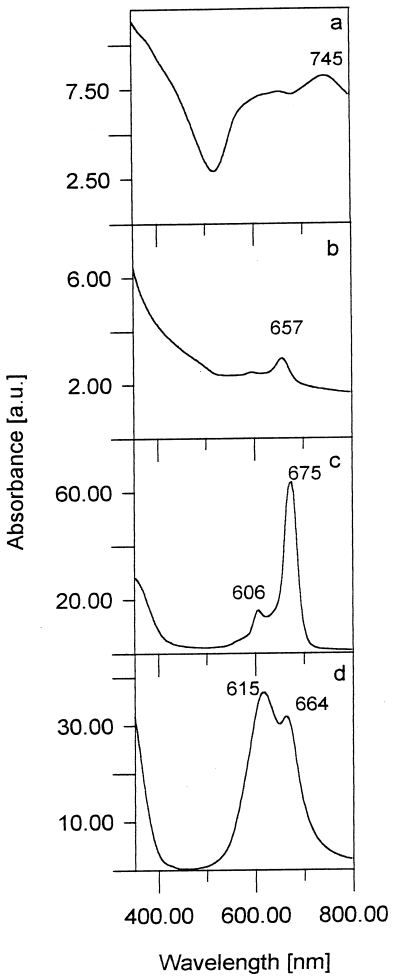


**Figure 2. Absorption spectra of phthalocyanines in DMSO.**

a) $H_2Pc$; b) CoPc; c) Solar Pc; d) Solar Pc in aqueous 5% DMSO. Concentrations of dyes $10^{-2}$ M.

In solvents used only Solar Pc exhibited measurable fluorescence (Fig. 3), but all dyes gave significant PAS spectra (Fig. 4). The Solar Pc fluorescence maximum is located at 688 nm. The Stokes shift between the main absorption maximum

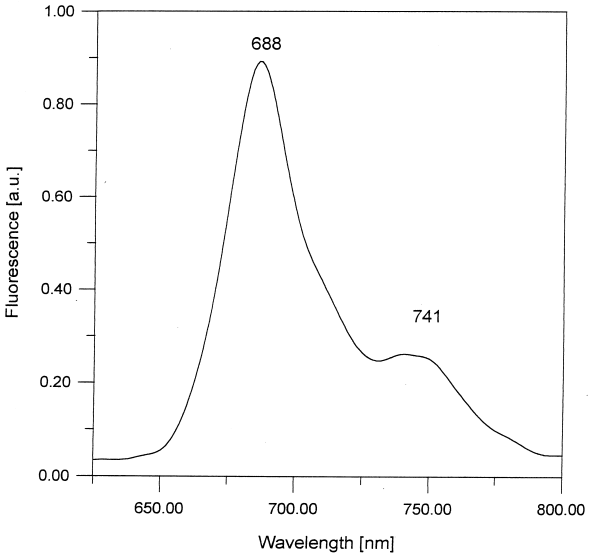


**Figure 3. Fluorescence spectrum of Solar Pc in DMSO.**

Excitation wavelength 607 nm.

(probably due to absorption of dye monomers) and the fluorescence maximum is only 13 nm.

Thermal deactivation (TD) can be usually calculated in arbitrary units from the PAS and absorption spectra. The investigated dyes exhibit different solubilities in DMSO so their TD can be only roughly evaluated. The shapes of the absorption and PAS spectra are different, especially for Solar Pc. This strongly suggests the presence of several dye forms, for example various dye aggregate in the incubation solvent. These forms can exhibit different yields of TD. Aggregation of dyes is know to change their photochemical properties and their uptake by cells [2, 12, 13, 18]. To confirm the supposition that the aggregated forms of the dyes are present in the cells studied dependence of PAS spectra on the frequency of light modulation was measured for $H_2Pc$ and Solar Pc in DMSO (Figs. 4a and 4b, respectively). The spectra in Fig. 4a have been normalized at 700 nm and those in Figs. 4b and 4c at 670 nm. The normalization was necessary to compare the shapes of the spectra because changing of the modulation frequency always changes the amplitude of the PAS. A similar set of measurements was repeated for the dye immobilized in a polymer film, because such samples are photochemically stable and several PAS spectra can be taken without destroying them. The results for the two media were similar

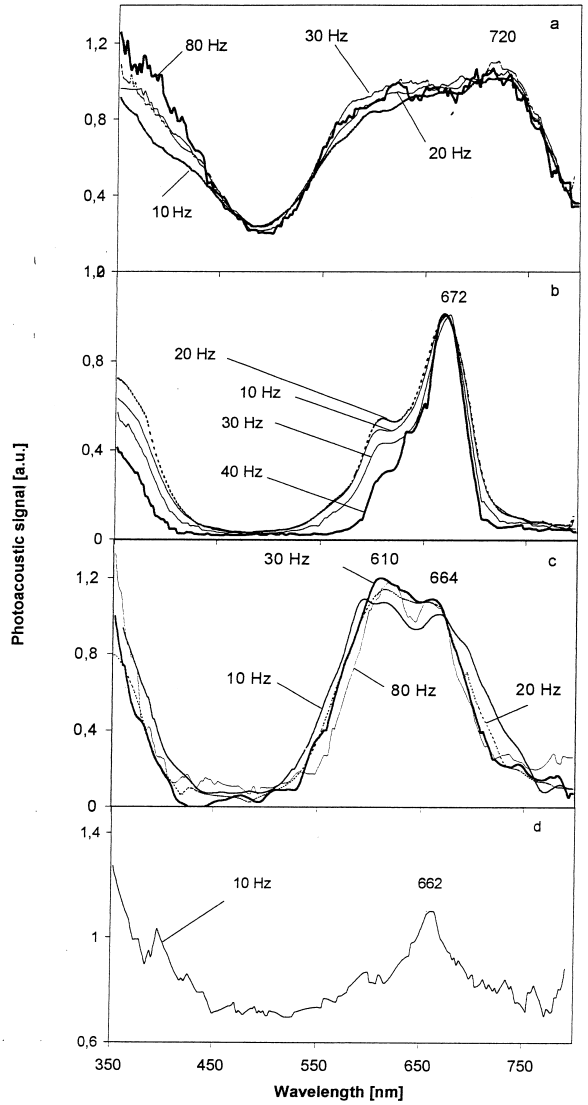


**Figure 4. Photoacoustic spectra of phthalocyanines in DMSO.**

a, $H_2Pc$, normalized at 700 nm; b, Solar Pc normalized at 670 nm; c, Solar Pc in 5% aqueous DMSO normalized at 670 nm; d, CoPc normalized at 670 nm. Frequencies of light modulation for PAS marked on curves.

(not shown). The shapes of the Solar Pc PAS taken at various light modulation were different (Fig. 4b). Smaller changes in the shapes of the PAS were observed for $H_2Pc$. By comparing the absorption of the Solar Pc (Fig. 2c) with the PAS (Fig. 4b) it can be seen that the main absorption maximum at about 670 nm converts less of the absorbed energy into heat than the shoulder at about 600 nm. The ratio of the main maximum to the shoulder changes from 2.4 for 10 Hz to 1.5 for 40 Hz. This result supports the supposition that

the Solar Pc absorption at about 600 nm is due to dye aggregates. The ratios of all the maxima are dependent on the modulation frequency (Table 1). This confirms the presence of forms with different thermal deactivation kinetics [18]. Figure 4c shows the dependence of the PAS on the frequency of light modulation for Solar Pc in 5% aqueous DMSO solutions. In this case the absorption from the aggregates dominates, so the change of the ratio of the maxima with frequency is less remarkable. It is the change in the shape of the PAS that suggests the occurrence of several types of dye aggregates. It is known [1] that the dye aggregates contribute to the $H_2Pc$ short wavelength absorption shoulder. It is likely that there may be some negative correlation between the TD in DMSO and the fluorescence yield. Non fluorescent dyes will probably have a much higher TD. From a crude evaluation it seems that strongly fluorescent Solar Pc exhibits a lower TD than $H_2Pc$ or CoPc which are non fluorescent in DMSO. Figure 4d shows the PAS of the CoPc solution in DMSO. Because of the low solubility of the dyes (Fig. 2b), the PAS signal is also low.

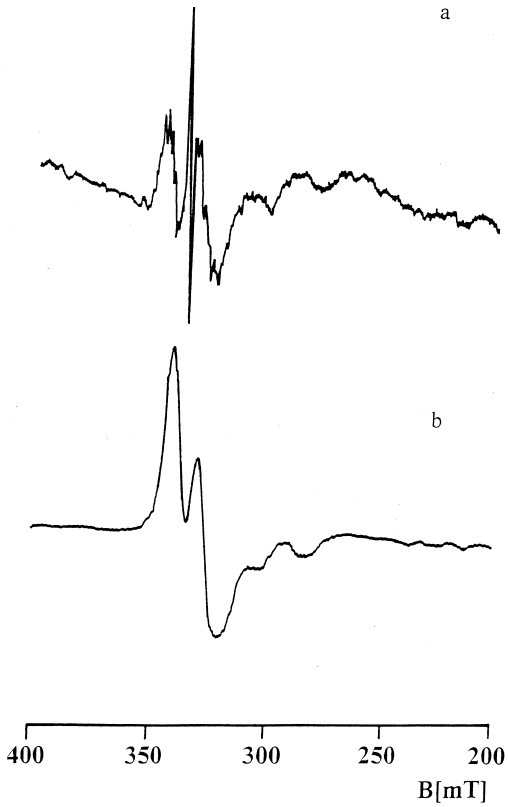


**Figure 5. EPR spectra of $H_2Pc$ (top) and Solar Pc (bottom) measured in DMSO in room temperature.**

Modulation amplitude 0.2 mT, microwave power 15 mW, microwave frequency 9.38833 GHz, B, magnetic induction.

Figure 5 shows the EPR spectra of Solar Pc and $H_2Pc$ in DMSO. The EPR signal of CoPc in DMSO in these conditions was not measurable. Crystalline CoPc occurs in three polymorphic forms [19]. One of them ($\beta$ form) was not detectable by EPR at room temperature whilst for the others ($\alpha$-form and $\gamma$-form) EPR signals were observed [19]. In our experiments a solution of CoPc was used so it is very likely that the interactions occuring in our samples were similar as for the $\beta$-form. Thus there would be greater contributions from orbital moments [5] and therefore one would not expect the EPR signal to be measurable at room temperature. Rollman & Iwamoto [20] observed an EPR signal in DMSO at room temperature for another CoPc compound ($Na_4CoPc$) but the signal had a low intensity.

Only $H_2Pc$ in DMSO at room temperature shows a stable free radical signal. This is evident from Fig. 5a which shows the strongest EPR line at 335 mT. For Solar Pc the free radical signal is observed neither in DMSO nor in PVA film. In all these cases the influence of $Cu^{2+}$ impurities can be expected to be giving different hyperfine structure intensities. The concentration of Solar Pc ($10^{-2}$ M) is sufficiently low so, that dipolar interaction between the $Cu^{2+}$ ions do not disturb the hyperfine structure of the $Cu^{2+}$ nuclei (Fig. 5b). A similar situation is observed for $H_2Pc$ in DMSO but in this case the dipolar interactions between $Cu^{2+}$ ions are weaker and the superhyperfine structure from the Pc nitrogen nuclei (structure in Fig. 1) is partially resolved (Fig. 5a). For the same dye concentration in DMSO the two metal-free Pcs (Solar and $H_2Pc$) show a differently resolved superhyperfine structure of N in Pc. Probably the peripheral substituents in these two Pcs make different contributions to the screening of unpaired electrons. The Solar Pc molecules which are embedded in PVA should be more separated or better screened because the superhyperfine structure of N nuclei in this Pc is very well resolved (Fig. 6).

It can be seen from Figs. 2–6 that the investigated dyes have very different spectral properties

in the same solvent. Changing of the solvent has also a remarkable influence. It is not simple to predict the behavior of dye molecules in complex biological samples. There are several general predictions concerning Pcs. It was suggested [21] that axially ligated Pcs were more likely to be useful in photodynamic therapy than peripherically substituted ones. Solar Pc is a typical peripherically substituted Pc molecule which we tested in this respect. We compared metal-free Pcs with CoPc. Metal-substituted Pcs are gener-

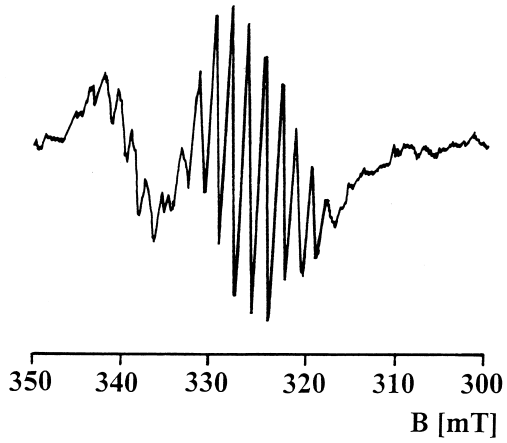


**Figure 6. EPR of Solar Pc in PVA film.**

Room temperature, modulation amplitude 0.2 mT, microwave power 1.5 mW, microwave frequency 9.35739 GHz, B, magnetic induction.

ally promising as sensitizers [22, 23]. However, in a Pc molecule Co behaves like a paramagnetic metal [1] and so it diminishes the lifetime of the Pc triplet state. Thus one can predict that CoPc will not be an efficient photosensitizer for either mechanism of sensitization (Type I – by direct interaction of CoPc in the triplet state with the cell, and Type II – by generation of a singlet oxygen) [24].

### Phthalocyanines in peripheral blood mononuclear cells

The effect of solvent, Solar Pc, activator (PHA) and incubation time on absorption spectra of PBMC is shown in Fig. 7a–e. Figure 7a shows the absorption spectrum of non stained PBMC in solution of NaCl in water, and Fig. 7b the spectra of the cells incubated in DMSO (curve 1) and in So-

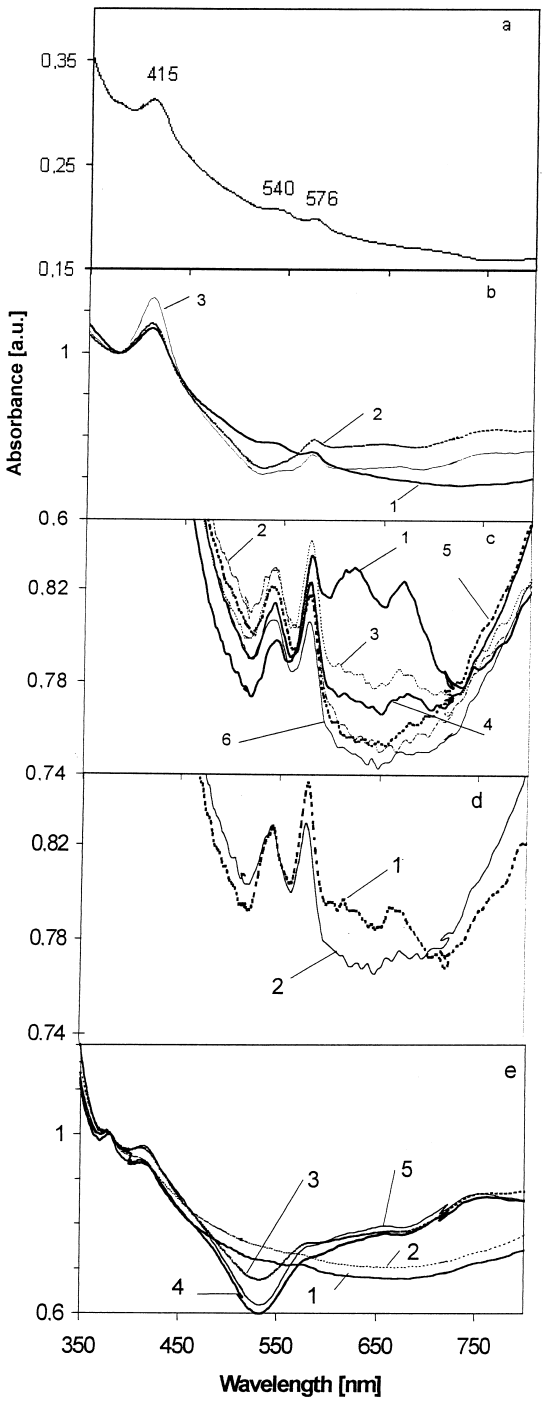


**Figure 7. Absorption spectra of peripheral blood mononuclear cells.**

a, PBMC in NaCl water solution. b, Incubated for 15 min, curve 1: in DMSO only; 2: in DMSO with Solar Pc and PHA, 3: in DMSO in Solar Pc. Normalized at 380 nm. c, Incubated for 1 h in DMSO; curve 1: Solar Pc and PHA, 2: PHA, 3: PHA+Solar Pc+serum (2%), 4: Solar Pc, 5: DMSO only, 6: Solar Pc+serum (2%). d, Incubated for 1 h in 5% aqueous DMSO; curve 1: Solar Pc+PHA+serum, curve 2: Solar Pc+serum. e, 15 min of incubation in DMSO curve 1: in DMSO + PHA, 2: in DMSO only, 3: with $H_2Pc$; 4: with $H_2Pc$ +PHA, 5: $H_2Pc$ +PHA+ serum.

lar Pc dye solution for 15 min (with PHA curve 2, and without PHA, curve 3). The presence of Solar Pc caused an increase in absorption in the 600–750 nm region (Fig. 7b). This increase was a little higher for stimulated cells than for the resting cells, but on 15 min incubation the incorporation of the dye was inefficient. After 1 h of incubation (Fig. 7c) the absorption with Solar Pc in the stimulated cells (curve 1) was much higher than for the resting cells (curve 4). It is evident from a comparison of curve 3 with curve 6 that addition of serum to the incubation solvent did not improve the incorporation efficiency. When either the activator was added without the dye (curve 2) or with DMSO only (without a dye or PHA) the spectrum in the dye absorption region was not much changed.

For PBMC incubation in 5% aqueous DMSO the incorporation of the dye and incorporation selectivity were better than for incubation in DMSO. Addition of serum slightly improved there parameters (curves 1 and 2 in Fig. 7d) both for the PHA stimulated cells and non stimulated cells.

The absorption of $H_2Pc$ in the region 550–750 nm was higher for the cells incubated with the dye than for unstained cells (Fig. 7e) even if the incubation time was only 15 min.

The dye concentration used in most of our experiments was $10^{-2}$ M. Blood serum, added to some sets of the samples to decrease the dye aggregation [2], improved the dye uptake in the samples incubated with a high water content, other wise the dyes were highly aggregated in such solutions. However serum addition had no effect on the samples incubated in DMSO (Fig. 7c).

The incorporation of $H_2Pc$ and Solar Pc dyes into the cells can clearly be seen from the difference absorption spectra (cells with the dye minus cells without the dye) presented in Fig. 8. By comparing panels 8a and 8b it can be seen that in both solvents the efficiency of Solar Pc incorporation into activated cells was higher than into the resting cells. This effect was stronger for DMSO than for DMSO with a large content of water. The incubation time had to be established separately for each Pc because it can vary considerably [25]. In our samples the cells became degraded when the

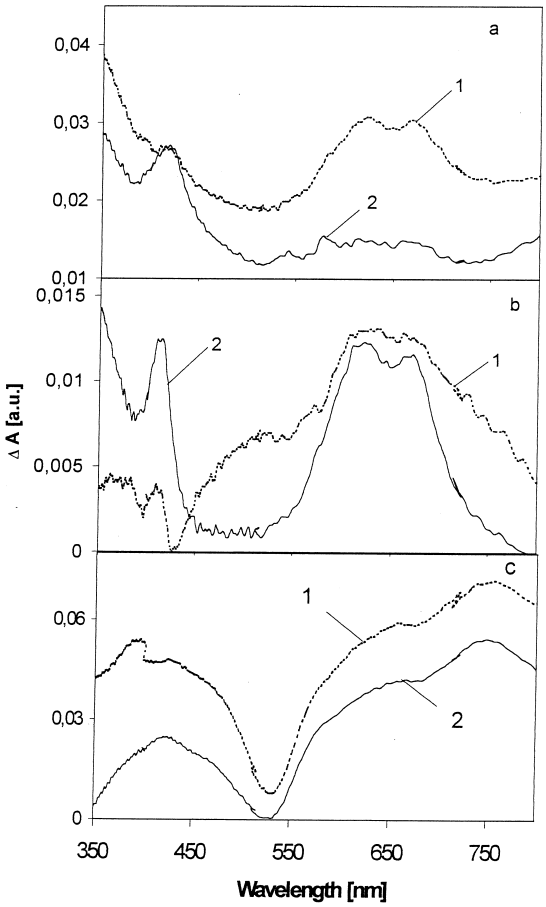


**Figure 8. The difference absorption spectra of PBMC.**

a, Cells in DMSO, incubation time 1 h; curve 1: Solar Pc+PHA ($A_1$) minus PHA only ($A_2$); curve 2: Solar Pc ($A_1$) minus cells only (A2); b, the same as a, only cells in 5% aqueous DMSO; c, incubation in DMSO, time 15 min: curve 1: with $H_2Pc$ and PHA ($A_1$) minus cells only ($A_2$); curve 2 ($A_1$) with $H_2Pc$ minus ($A_2$) cells with PHA only.

incubation time was increased to 6 h (not shown). For the 15 min incubation time the uptake effects were low, with the optimal time being 1 h. A decrease in the dye concentration diminished the number of incorporated dye molecules (not shown) but this also was dependent on the dye solubility. Also in the case of $H_2Pc$ the leukocytes activated with PHA were more strongly stained than the non-activated cells (Fig. 8c).

The selectivity in the dye uptake is a promising property of both $H_2Pc$ and Solar Pc. The absorption spectra of the stained cells can be perturbed by light scattering. It was therefore expected that the additional information on this point could be

**Tabele 1. Ratio of PAS amplitudes measured at various wavelengths (nm) and frequencies of light modulation (f) for Solar Pc in DMSO (accuracy ±0.1)**

| f Hz | Amplitude ratio at given wavelengths | | |
|---|---|---|---|
| | 616 nm/370 nm | 616 nm/676 nm | 370 nm/676 nm |
| 10 | 1.4 | 2.4 | 1.7 |
| 20 | 1.4 | 2.5 | 1.8 |
| 30 | 1.6 | 2.0 | 1.2 |
| 40 | 1.7 | 1.5 | 0.9 |

obtained from PAS spectra and flow cytometry measurements for the cells which were only lightly stained.

Cytometric measurements enable the observation of very weak fluorescence from dyes incorporated into cells. This fluorescence is superimposed on the cell emission at the applied excitation wavelength in observation spectral regions. Human leukocytes exhibit several bands of inherent fluorescence which is strongly dependent on the chemical treatment of the cells [26]. The mean channel fluorescence measured through a green filter is a superposition of the cell and the dye fluorescence. Monocytes (M) have a higher fluorescence intensity than lymphocytes (L) (Table 2).

As measured by flow cytometry, addition of $H_2Pc$ or Solar Pc changes the fluorescence of cells, whereas addition of PHA only or prolonged incubation of the cells in DMSO influence the fluorescence intensity (Table 2). In some cases, in contradiction with the absorption results, the fluorescence emission was higher for resting than for stimulated cells. Serum addition also changed the emission. It is known that the fluorescence yield of dyes is much lower in cells than in solutions [14]. Therefore staining of the resting or activated cells with Solar Pc or $H_2Pc$, can cause only a slight increase in cell fluorescence. The dye fluorescence yield is dependent on the dye molecules interaction with the environment. The perturbation caused in the cells by the activator can differ from that caused by DMSO. As a result, the fluorescence yields of the cells and of the dye can be changed differently by perturbation of the cell structure. This could explain the discrepancy between the absorption and fluorescence data.

In the case of CoPc the fluorescence intensities of stained and non stained cells were identical. This could be due to the very low fluorescence yield when this dye is incorporated into cells. However, the PAS and absorption spectra suggest that this dye is practically not incorporated into cells.

It is clear from the absorption spectra of the cells with and without the dye that the properties

**Table 2. Mean channel cytometer fluorescence intensity of leukocytes (L) and monocytes (M) in DMSO§ or in 5% aqueous DMSO***

| Sample | Time | C | C+PHA | C+PHA+D | C+D | C+D+S | C+D+PHA+S |
|---|---|---|---|---|---|---|---|
| M $H_2PC$§ | 15 min | 38.8 | 33.3 | 42.3 | 31.8 | 35.5 | 40.6 |
| L $H_2PC$§ | | 21.6 | 16.1 | 20.5 | 14.1 | 18.2 | 19.2 |
| M Solar§ | 15 min | 42.2 | 48.0 | 53.6 | 51.3 | 46.1 | 51.5 |
| L Solar§ | | 25.7 | 26.1 | 31.3 | 32.6 | 29.1 | 29.7 |
| M Solar§ | 1 h | 71.1 | 64.4 | 61.2 | 71.9 | 61.3 | 76.4 |
| L Solar§ | | 42.9 | 29.3 | 31.7 | 52.7 | 35.1 | 34.3 |
| M Solar* | 1 h | 58.6 | 64.0 | 65.3 | 62.7 | 70.4 | 67.5 |
| L Solar* | | 33.1 | 30.5 | 31.3 | 33.3 | 37.4 | 31.2 |

C, cells; D, dye; PHA, activator; S, serum. Concentration of dye in incubation solvent $10^{-2}$ M. Time, time of incubation at 37°C.

of stained PBMC are influenced by both factors: the absorption of added dye and the perturbation of the cell material.

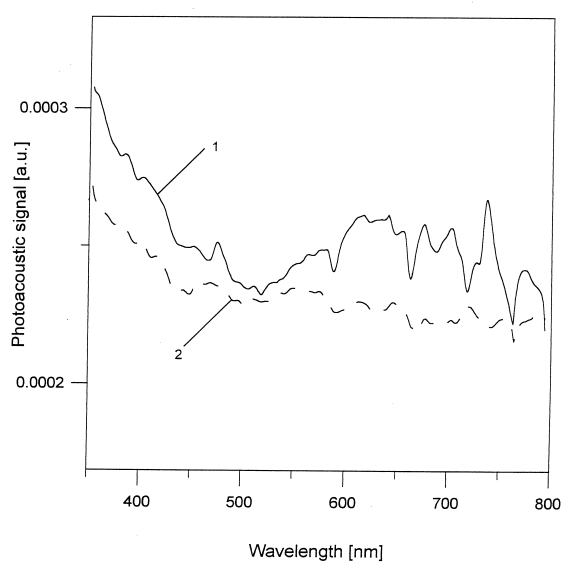


**Figure 9. Photoacoustic spectra of cells incubated 1 h in 5% aqueous DMSO.**

Curve 1: Solar Pc + PHA; curve 2: PHA with cells only.

It has been shown previously [6] that activators perturb the membrane structure. Now it can be seen that also addition of dye influences the properties of leukocytes.

It is apparent from the comparison of the cytometric results for the investigated dyes that it is not possible to evaluate the dye incorporation on the basis of cytometric measurements only. The photoacoustic spectra of the stained cells are partially due to thermal deactivation (TD) occurring in the cell material. The contributions from the dye are also evident in the PAS (Fig. 9) taken for Solar Pc in 5% aqueous DMSO in which the dye is not incorporated very efficiently. Some results concerning TD of PBMC are gathered in Table 3. The PHA addition increases the amplitude of the PAS. When the dye is added at a low concentration it changes the PAS less than activator addition. The PAS of the cells in DMSO is different than that of the same sample in aqueous NaCl. The large differences between the values of TD in these two solvents cannot be explained by the different thermal properties of these solvents, so they must be caused by the denaturation of cells by DMSO.

For Solar Pc the highest value of TD is observed for the cells incubated with PHA, dye and serum especially in the 570 nm and 672 nm regions in which the dye is absorbing. High values are also observed for cells with PHA but without the dye. This shows that the contribution to the PAS from denatured cells is also important. The cells incubated with both PHA and the dye show lower TD than cells with PHA only. This means that the dyes affect the action of PHA. For $H_2$Pc, PHA + the dye, the sample gives a much higher PAS

**Table 3. Thermal deactivation of leukocytes (L)**

| D;C | $\lambda$ [nm] | Time | L‡ | L | L+S | L+PHA | L+D | L+PHA+D | L+S+PHA+D |
|---|---|---|---|---|---|---|---|---|---|
| Solar§ | 415 | 1 h | 0.23 | 0.37 | 0.40 | 0.76 | 0.41 | 0.58 | 0.71 |
| $10^{-2}$ M | 570 | | 0.24 | 0.50 | 0.53 | 0.84 | 0.54 | 0.63 | 1.03 |
| | 672 | | 0.24 | 0.56 | 0.55 | 0.77 | 0.53 | 0.59 | 0.92 |
| $H_2$ Pc§ | 415 | 15 min | – | – | – | 0.64 | 0.49 | 0.68 | – |
| $10^{-4}$ M | 570 | | – | – | – | 0.59 | 0.54 | 0.86 | – |
| | 672 | | – | – | – | 0.70 | 0.04 | 0.70 | – |
| Solar* | 415 | 15 min | – | – | – | 0.15 | – | 0.24 | – |
| | 570 | | – | – | – | 0.20 | – | 0.22 | – |
| | 672 | | – | – | – | 0.19 | – | 0.24 | – |

D, dye; C, dye concentration in incubation solvent; S, serum; ‡L, leukocytes in NaCl water solution; *in 5% aqueous DMSO; $\lambda$, wavelength of observation; Time, time of incubation at 37°C.

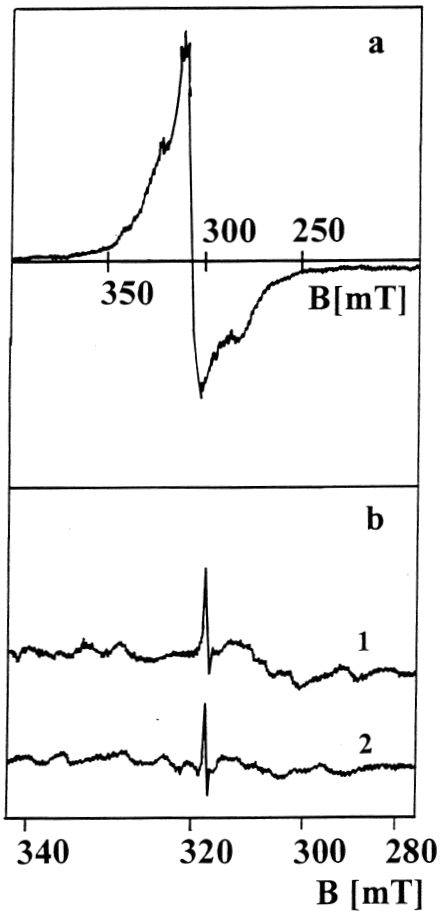


**Figure 10. EPR spectra at 77 K.**

a, Solar Pc in 5% aqueous DMSO. Microwave frequency 8.8763 GHz; modulation amplitude 0.4 mT; microwave power 1.5 mW; B, magnetic induction. b, Leukocytes stained by Solar Pc: curve 1, stimulated cells, curve 2, resting cells, microwave frequency 8.8766 GHz, others parameters as in a.

value than the cells with PHA only, but exclusively in the 570 nm region. For CoPc, the values of the PAS amplitude for stained and non stained cells are identical, showing once more that the uptake of this dye into the cell is very low (not shown).

The change in the TD of the cell material caused by DMSO and PHA complicates the interpretation of the PAS data. However the difference in TD both in the region of a strong dye absorption and outside this region suggests that the contributions from the dye are measurable. When the dye is efficiently incorporated into the cells the PAS method seems to be more useful than cytometric data.

The EPR spectra of the stained PBMC do not exhibit the characteristic signal observed for dyes at room temperature, therefore some spectra were measured at of 77 K. The EPR spectra of some solutions of the dyes were also taken at this low temperature. Under such conditions CoPc exhibited a very intensive line (not shown), similar to that observed for $H_2$Pc and Solar Pc (Fig. 5). This result supports our supposition that this line was due to a Cu admixture.

The EPR spectrum of Solar Pc in 5% aqueous DMSO measured at 77 K (Fig. 10a) shows a much higher signal than that observed at room temperature (Fig. 5).

Figure 10b shows the EPR spectra measured at 77 K for the leukocytes stained by incubation in Solar Pc solution in DMSO with water. The stimulated cells exhibit a broad signal in the 300–310 mT region, which is higher than the signal for resting cells. This shows that Solar Pc is more efficiently incorporated into stimulated than into resting cells.

The cells incubated in CoPc in DMSO did not give any signal which could be due to Co. This observation supports all other results taken for this dye which show a very low incorporation of CoPc into the cells.

## CONCLUSIONS

The investigated dyes differ strongly in their spectral properties, their yield of incorporation into cells and their influence on inner cell structure as demonstrated by changes in TD and fluorescence.

The fluorescence of the investigated Pcs in the cells is very low so these dyes can be applied in photodynamic therapy but not in cancer diagnosis. The perturbation of TD and fluorescence of normal (resting) cells caused by these dyes suggests that they may be toxic. Thus, the action of Pcs should be carefully investigated in different tissue samples.

Solar Pc seems to be the most promising of the investigated dyes because it is fluorescent and is selectively incorporated into normal and neoplastic (malignant) cells.

The applied combination of different spectral methods i.e. measurements of absorption, PAS,

EPR and fluorescence provides of information about the possibility of using these dyes in photodynamic therapy.

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