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Electric depolarization of photosensitized cells: lipid vs. protein alterations

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We have monitored several photosensitized reactions in proteins, liposomes and cells under similar conditions. We found that the depolarization of K^+-diffusion potential of liposomes or the leakage of an entrapped molecule, calcein, progress at a much slower rate than the photosensitized damage to proteins and the photosensitized killing of bacterial and leukemic cells. X-ray microanalysis revealed that upon light exposure of HP-treated leukemic cells and bacteria, they totally lost their cellular potassium. We deduce that the direct photosensitized oxidation of lipid components cannot cause the depolarization of cells, which in turn could be responsible for their death. A photosensitized damage to protein sites in the cell, probably in the membrane, is a more likely reason for the depolarization, the loss of potassium ions and cell death that is caused in light-activated photodynamic processes.

Introduction

Tumor necrosis by the photodynamic action of photosensitizers is now a well established phenomenon [1]. It is currently tested as an experimental modality for treating light-accessible solid tumors. The issue of the primary mechanism of necrosis is being debated, whether it is a direct damage to the cancer cells or whether the damage occurs mainly to the endothelial cells of blood vessels in the tumor and in vessels leading into it [2]. In either case, however, a basic question arises as to the primary target for photooxidation within the cell, which leads eventually to a lethal damage to that cell.

The immediate lethal action of photodynamic therapy (PDT) in the cell depends on its concentration in the various cellular organelles and compartments and on the relative photochemical reaction rate of singlet oxygen with different molecular targets. Anionic lipophilic sensitizers such as hematoporphyrin derivative (HPD) or Photofrin II (PF-II) partition into the cytoplasmic membrane as well as to intracellular membranes [3]. Thus, the injury which is caused to encapsulating membranes leads to damage in activated transport mechanisms, depolarization of the resting potential and leakage of molecules across the membranes [4,5].

In this study we wanted to estimate the relative feasibility of some of the above mechanisms. In particular, we were interested in assessing the importance of the depolarization of a cell's electric field in the process of photodamaging the cell. Such depolarization accompanies the death of a cell. It can be a direct cause of the death if the membrane is pored 'mechanically' by extrinsic channels or carriers, by chemically damaging the lipids or by chemically damaging membrane-bound protein systems such as pumps. We thus studied the photodamage to proteins and amino acid residues and the photosensitized injury to intact phospholipid vesicles in comparison with the photodynamic action on intact cells. The goal of this study was to assess the relative efficiency of damage to protein sites vs. damage to the lipid component of vesicles, as manifested in their leakage of entrapped species.

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Abbreviations: BR, bacteriorhodopsin; BSA, bovine serum albumin; CFU, colony-forming units; DMI, 1,2-dimethylindole; FELC, Friend erythroleukemic cells; HP, hematoporphyrin; HPD, hematoporphyrin derivative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; O_2 ($^1\Delta_g$), molecular singlet oxygen; PDT, photodynamic therapy; PF-II, Photofrin II; PM, purple membrane.

Materials and Methods

Chemicals

Hematoporphyrin (HP) dihydrochloride (Aldrich) was kept in a stock solution in buffer (pH = 6.8). PF-II was a gift from Photomedica (Raritan, NJ) and was taken from a concentrated stock solution in water, (pH = 6.8). Calcein, 1,2-dimethylindole (DMI), bovine serum albumin (BSA), poly(L-lysine), valinomycin and egg-yolk lecithin were purchased from Sigma (St. Louis. MO). The lecithin's composition was 34% C_{16} chains, 12% C_{18}, 48% unsaturated chains. The potentiometric dye probe diS-C_2(5) was purchased from Molecular Probes and stocked in ethanol.

Preparation of lecithin liposomes

Standard lecithin liposomes were prepared by evaporating the chloroform solvent, forming a layer of the lipid at the bottom of a test tube, re-dissolving in ethyl ether and re-evaporating to dryness. After addition of the aqueous solution the sample was vortexed for 3 min and then sonicated for 5 min by a probe sonicator (MSE, Crawley, UK) until a clear solution was obtained.

Preparation of reconstituted bacteriorhodopsin vesicles

The isolation and purification of purple membrane (PM) was carried out according to the method described by Oesterhelt and Stoeckenius [6]. The PM fragments were then reconstituted into lecithin liposomes by co-sonicating a lecithin liposomes' suspension with the PM fragments under nitrogen, by a probe sonicator, for 15 min [7]. The concentration of BR in the sample was set to 3 μM, using the absorbance value $\epsilon_{568} = 63\,000\ M^{-1}cm^{-1}$ [8,9]. The concentration of the lipid was 5 mg/ml. At these concentrations, where the molar ratio of lipid/BR is more than 1000, the two-dimensional crystalline structure of the PM fragments is dispersed into a monomeric molecular dispersion of bacteriorhodopsin (BR) [10]. The reconstitution of BR was verified by two different ways: by checking its characteristic monomeric CD band [11] and by performing a proton pumping experiment in which the reconstituted BR was illuminated with a 250 Watt slide projector lamp and the increase in the solution's pH was measured. The reconstituted liposomes were incubated with the porphyrin sensitizer and illuminated by the HeNe laser. At the indicated time intervals during the photosensitization, the laser irradiation was interrupted to measure the absorption spectrum of the BR's retinal chromophore.

Liposomes for diffusion potential experiment

Suspensions of liposomes at 5 mg lipid/ml were prepared in 0.2 M KCl and HP or PF-II were added to the suspension to a 10 μM concentration. To test the leakiness of the liposomes during the irradiation at various time intervals, 10-μl aliquots of the liposomes were added to 1 ml of 0.2 M NaCl solution containing the potentiometric dye diS-C_2(5) (1 μM). This 100-fold dilution of the extra-liposomal K^+ generates a 118 mV K^+-diffusion potential, inside negative, when valinomycin (Sigma) is added to a final concentration of 1 nM. The decrease in the diS-C_2(5) fluorescence due to the induced potential was then measured in the fluorimeter in a time-drive mode. The excitation wavelength was 645 nm and the emission wavelength was 670 nm.

Liposomes for calcein release experiment

Calcein-containing vesicles were prepared in 60 mM calcein (self quenching concentration), at pH 7.2 (10 mM Hepes, Sigma). The non-encapsulated calcein was removed from the liposomes' suspension by the mini-column centrifugation technique [12]. The mini-columns were loaded with Sephadex G-50 (Pharmacia LKB Biotechnology) and then equilibrated with Hepes (10 mM), NaCl (0.15 M), pH 7.4. 50 μl of the liposomes' suspension were passed through the column, resulting in a 10-fold dilution. 10-μl aliquots of the calcein-containing liposomes were added to 2 ml of calcein-free liposomes (2 mg/ml). The final concentration of the calcein-labeled liposomes in the sample was 2 μg/ml. During the irradiation the time-course of the fluorescence intensity at 520 nm was monitored by excitation at 486 nm.

Photosensitization of bacterial and animal cells

Staphylococcus aureus cells were recovered from clinical material, and grown overnight in Brain-Heart Infusion broth (Difco, USA) then transferred into fresh medium. The bacteria were grown with aeration at 37°C in pH 6.5. For photosensitization experiments, the cells that were grown without the sensitizers were transferred to a 10 mM phosphate buffer (pH = 6.5) ($1 \cdot 10^8$ cells/ml) where they were incubated with the porphyrins (10 μM) for 2 h. We measured the survival rate during the photosensitization by counting colony-forming units (CFU) on nutrient agar plates (Difco, USA) after incubation for 24 h at 37°C. The Survival Fraction was N/N_0, where N_0 is the number of CFU/ml of the untreated culture and N is the number of CFU/ml of the treated cultures, after the indicated laser-irradiation times. In some experiments we monitored the fluorescence of the bacterial tryptophans during the photosensitization (excitation at 275 nm, fluorescence intensity measured at 330 nm).

Friend erythroleukemic cells (FELC), line 745, subclone 21 were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Gibco, Grand Island, NY) in a humidified incubator enriched with 10% CO_2 at 37°C. The cells were sub-

cultured twice a week by re suspension in fresh medium at a concentration of approx. $5 \cdot 10^5$ cells/ml. Cells grown under the above conditions were collected and washed in 0.1 M phosphate-buffered saline (pH 7.2). They were resuspended in fresh media, without serum, to a concentration of $1 \cdot 10^8$/ml in a buffered solution, and were incubated with 10 μM HP from stock solution. Survival rates were determined by the colorimetric method of Mosmann [13]. Briefly, a colorless tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added at a concentration of 0.5 mg/ml to cells suspended in medium and incubated at 37°C for 4 h. MTT was cleaved enzymatically in mitochondria and the water-insoluble reaction product, a dark blue formazan, was solubilized by 100 μl of 0.04 M HCl in isopropanol. Optical density was measured at 570 nm, and the percentage of viable cells calculated. The linearity of this method extends over a wide range of cells. Dead cells (treated by KCN) were completely negative to production of the blue formazan.

Irradiations

The irradiations were carried out with a HeNe laser having an output power of 15 mW at 632.8 nm. The beam's power on the sample's surface was measured with a Newport Corporation optical power meter (model 835). The sample (2 ml solution or suspension, in a 1×1 cm cuvette) was stirred magnetically to obtain uniform irradiation of the whole sample, with the laser beam traversing the 2 cm height of the sample. In all the studied cases, 10 μM HP or PF-II were used.

Spectroscopic measurements

The fluorescence intensity, excitation and emission spectra and fluorescence time-drives were measured on a Perkin-Elmer digital fluorimeter type MPF-66, which was controlled by a Data Station 7500 computer. Absorption spectra were measured on a Varian DMS 200 spectrophotometer and when necessary, were transferred to an IBM PC for further processing. CD spectra were measured on a Jasco J-500A spectropolarimeter under nitrogen, in a capped quartz optical cell with 10 mm path length at 23°C in the range of 450 to 640 nm.

X-ray microanalysis of cells

X-ray microanalysis was performed on each of the three experimental cell types. At the end of the porphyrin incubation interval, the cells were rinsed, resuspended in Dullbeco's phosphate-buffered-saline and attached to Thermanox plastic coverslips (Nunc, Nupervile, IL) which had been previously treated with poly(L-lysine). The cell monolayers on the cover slips were placed in multiwell dishes filled with the buffer and were illuminated for the required intervals. Thereafter, the attached cells were quickly rinsed by dipping them in 0.15 M ammonium acetate (pH 7) for 2–3 s. The rinsed cells were rapidly frozen with liquid nitrogen, freeze-dried at −80°C, and then coated with a layer of carbon [14]. X-ray microanalysis was performed on 50 cells of each sample using an eXL Link system attached to a Jeol 840 scanning electron microscope. Quantitation of the elements in the cells was performed using the Link ZAF-P/B program and a comparison was made with the control cells.

Results

Leakage of liposomes under photosensitization

We investigated the effect of photosensitization of natural egg phospholipids by PF-II and HP, on the intactness of the liposomes. We thus studied the effect of sensitization on the ability of the lipid bilayer to sustain an electric potential difference ($\Delta\psi$) and a chemical gradient of a dye, calcein. For the electric potential study, we used the potentiometric dye diS-$C_2(5)$, whose fluorescence decrease of about 40% per 100 mV (inside negative), is one of the strongest responses known among the potentiometric dyes [15]. The cross-membrane K^+-diffusion potential was induced in the liposomes by valinomycin. In Fig. 1 we show the effect of formation of the diffusion potential on the fluorescence of diS-$C_2(5)$. By following the fluorescence intensity of the dye after the addition of valinomycin (the lowest plateau), we found that the potential leaked by less than 5% over 20 min. Thus, a sample of liposomes in KCl, with PF-II or HP, was

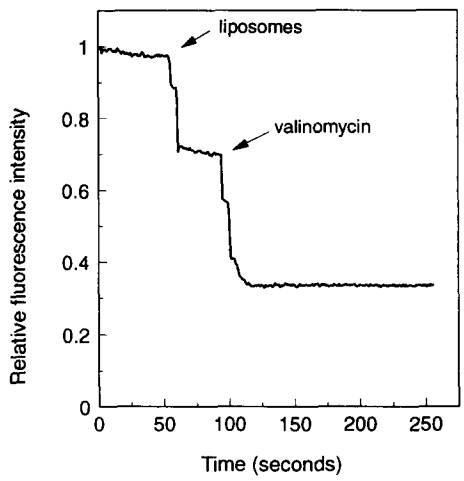

Fig. 1. Fluorescence monitoring of K^+-diffusion potential on liposomes. The initial part of the trace is the fluorescence level of the dye diS-$C_2(5)$, 1 μM in 0.2 M NaCl solution. Liposomes from a suspension in 0.2 M KCl were diluted (1:100), to a final concentration of 50 μg/ml, into the above solution of the dye. Valinomycin (1 nM) was then added. The fluorescence was excited at 645 nm and measured at 670 nm.

illuminated, and at constant time intervals the irradiation of the sample was interrupted and 10-μl aliquots were taken out. The aliquot was diluted into a NaCl solution containing the potentiometric dye, and the extent of the fluorescence decrease due to valinomycin-induced K^+ potential, was measured. This decrease in the fluorescence, which represents the extent to which the liposomes can sustain a gradient of K^+ ions, is shown in Fig. 2 as a function of the irradiation time, as a curve marked $\Delta\psi$. As can be seen, no significant change in the ability of the liposomes to hold a K^+-diffusion potential due to photosensitization by PF-II or HP, was observed over 2 h.

We also checked the effect of photosensitization by PF-II on the ability of the liposomes to maintain an entrapped concentration of calcein under the same irradiation fluences and the same concentration of PF-II. No leakage of entrapped calcein was observed over a period of 1 h.

To contrast these results of liposomes' damage and leakage, we shall now present our results on the photodamaging effect of the same concentrations of sensitizers and under the same illuminations conditions, of *S. aureus* bacterial cells and Friend erythroleukemic cells.

Photosensitization of S. aureus cells

We have shown before [16,17] that photosensitization of Gram-positive bacteria can be efficiently achieved, following the uptake of the porphyrins by the cells. The partitioning of porphyrins such as deuteroporphyrin, HPD or PF-II reaches saturation after just a few min. Thus, after 1 h incubation, HP has reached full equilibrium between the cells and the buffered

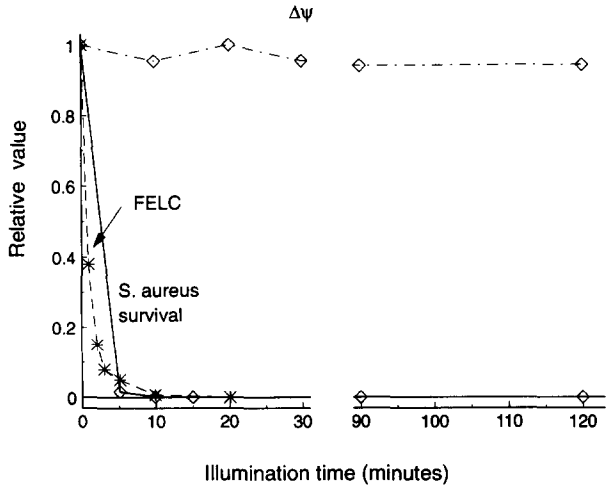

Fig. 2. Time-dependent decrease in several parameters in the process of photosensitization of liposomes and cells, by 15 mW HeNe laser illumination. The figure shows the extent of K^+-diffusion potential sustained by liposomes ($\Delta\psi$), and the survival ratio of Friend erythroleukemic cells and *S. aureus* bacteria after the indicated illumination periods.

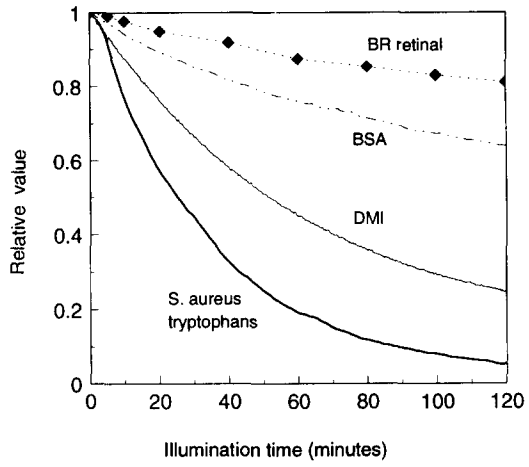

Fig. 3. Time-dependent decrease in several parameters in the process of photosensitization of protein model systems, by 15 mW HeNe laser illumination. The figure shows the photodamage of 1,2-dimethylindole (DMI), of retinal in bacteriorhodopsin, and of tryptophans in BSA and in *S. aureus* bacteria.

solution and more than 90% of it was bound to the cells. We followed the fluorescence intensity of the bacterial tryptophans in the cells' suspension, while it was illuminated by the laser. From the time-course of this measurement, shown in Fig. 3, we calculated a kinetic time constant of 37.7 ± 0.7 min. We resolved an additional, residual fluorescence component, which contributed about 30% of the initial intensity, and which decreased with a time constant of > 8 h. This component arises probably from tryptophans which are not accessible to singlet oxygen or from other cellular fluorophores.

In another experiment we employed an identical sample of *S. aureus* cells, incubated with HP and illuminated with the same laser. At different time points during the illumination we measured the survival rate of the cells. The results, shown in Fig. 2 and denoted as *S. aureus* survival, demonstrate more than 99% photodamage within 5 min of illumination.

Photosensitization of human Friend erythroleukemic cells

We used Friend erythroleukemic cells as a model for photosensitization in vitro by our various photosensitizers. The follow up of the photodynamic effect of HP on the survival rate of these cells, under equal conditions of sensitizer's concentration and laser intensity as in all the previous cases, is shown in Fig. 2, denoted as FELC. As can be seen, the mitochondrial activity measured by the MTT enzymatic assay decreased rapidly during the first 10 min of the illumination. The mitochondrial damage can be considered as an accurate method for cell viability, since it reflects the cellular capacity to produce ATP. The MTT method reflects a specific protein damage and therefore it can

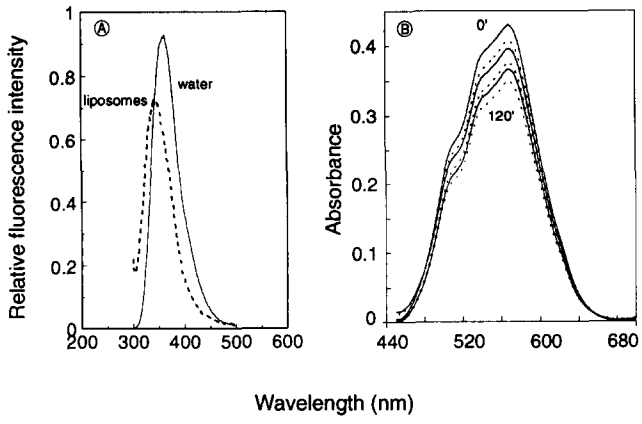

Fig. 4. (A) Fluorescence of 1,2-dimethylindole in water and in liposomes. DMI's concentration is 10 mM, the concentration of liposomes is 2.5 mg/ml. Excitation is at 292 nm. (B) Absorption spectra of BR reconstituted in liposomes, photosensitized by PF-II at times: 0, 20, 40, 60, 80 and 120 min. The initial concentrations of BR and PF-II were 3 μM and 10 μM, respectively. The concentration of the lipid was 5 mg/ml. A 15 mW HeNe laser was used as the irradiation source for the photosensitization.

be considered as another example for protein alteration during photosensitization.

Photosensitization of dimethylindole

We used 1,2-dimethylindole (DMI), incorporated in liposomal membranes, as a simple hydrophobic model for the tryptophan residue of membrane proteins. Photo-oxidation of indoles has received much attention, because of their possible role as targets during photodynamic action [18–20]. The kinetics of the photosensitization reaction of DMI can be used as a reference to the photosensitization of tryptophan residues in a membranal protein.

The fluorescence emission spectra of DMI in water and in lecithin liposomes are shown in Fig. 4A. As can be seen from the figure, the emission peak shifts from 362 nm to 342 nm upon partitioning into the liposomes. The fluorescence intensity of DMI at 325 nm, excited at 262 nm, was employed to evaluate its binding constant to the liposomes by a spectroscopic titration technique [21], and was 3.97 (mg lipid/ml)$^{-1}$. Based on this number, it can be calculated that about 90% of the DMI which is present in a 2 mg/ml liposomes' suspension resides in the lipid environment. Moreover, when the fluorescence of DMI was monitored at 325 nm in a photosensitization experiment by HP, the unbound, aqueous, DMI contributed only about 5% to the measured fluorescence. This is reflected in our time-drive plot merely as a non-zero final value to which the fluorescence intensity approaches asymptotically at long times.

The real-time fluorescence disappearance of DMI upon sensitization by PF-II is shown in Fig. 3. The reaction followed a first order rate law with a time constant of 1.26 ± 0.10 h. Since the lifetime of singlet oxygen in water is shorter than in organic solvents and in the membranal matrix [22,23], one can safely assume that practically all of the DMI molecules photosensitized by PF-II are membrane-bound. No changes in the fluorescence intensity of DMI were observed in the absence of sensitizer or when the sample was flushed with nitrogen and sealed with a glass cover prior to illumination.

Photosensitization of bovine serum albumin

Among all the serum proteins, albumin binds a significant share of the administered porphyrin dose. In this study we used BSA as a protein model, and we determined the photosensitization kinetics of the BSA's tryptophan residues by PF-II as well as its binding affinity to BSA.

The fluorescence emission spectrum of PF-II in PBS buffer (pH 7.2) exhibits the common shift of the main fluorescence band from 616 nm to 629 nm, upon binding to BSA, indicating that PF-II is in a hydrophobic site [16,24]. With these spectral features, we evaluated the binding constant of PF-II to BSA by the titration method [21] as 14.9 ± 0.10 (mg BSA/ml)$^{-1}$, or $1.01 \cdot 10^6$ M^{-1} taking the molecular weight of BSA as 68000. With this binding constant, about 90% of the sensitizer is bound to BSA at a latter's concentration of 0.5 mg/ml.

We measured the kinetics of the photodamage to the protein's tryptophans by PF-II, by monitoring the decrease in the tryptophans fluorescence at 340 nm. The kinetic trace, which is shown in Fig. 3 and denoted as BSA, followed a first order law with a time constant of 8.68 ± 0.45 h.

Photosensitization of reconstituted bacteriorhodopsin

In the native purple membrane of *Halobacterium halobium*, BR is organized in a two-dimensional hexagonal lattice of protein-trimers. Such organization gives rise to a characteristic CD signal due to the interactions between protein chromophores. The purple patches can be reconstituted into liposomes, and a similar protein arrangement and CD spectrum is observed in the reconstituted bacteriorhodopsin, below the phase-transition temperature of the bilayer. Upon increasing the lipid/BR ratio in reconstituted BR, the excitonic structure in the CD spectrum disappears and a single positive band appears [10,11]. This positive-only CD band indicates that the BR molecules are dispersed in the membrane as monomers. In this study we were interested to disperse the protein patches in which the lipid content is small, so that the reconstituted lipid phase would be more homogeneous in terms of both BR as well as PF-II localization. At a lipid/BR ratio of (5 mg/ml)/(3 μM), i.e., a molar ratio of about

2000, we obtained a single symmetrical positive CD band indicating the disappearance of the trimeric structure. Under these conditions we also observed an increase of 0.08 pH units in the liposomes' suspension upon illumination with white light, indicating inward proton pumping.

Next, we measured the photosensitization of the retinal residues of BR, by using PF-II as a photosensitizer. Some of the absorption spectra are shown in Fig. 4B, and the time-course of the decrease in BR's absorption at the retinal chromophore's band at 568 nm is shown in Fig. 3. We found that the retinal photosensitization kinetics followed a first-order reaction law with a time constant of 9.36 ± 2.08 h. No changes in the absorption spectrum were observed in the absence of sensitizer or when the sample was saturated with nitrogen during the photosensitization.

X-ray microanalysis of photosensitized cells and bacteria

X-ray microanalysis, combined with scanning electron microscopy, provides a tool for elemental analysis of individual cells fixed by quick freezing in liquid nitrogen. Thus, the kinetics of elemental and ionic changes in the cells during short intervals of photoactivation was possible. Figs. 5A and 5B show the photodynamic effect on K^+ depletion from FELC and *S. aureus* bacteria during 10-min light irradiation. The spectra expose only the intra-cellular elements and not outside contaminants, since the cells were rinsed thoroughly with ammonium acetate buffer prior to liquid nitrogen freezing. The results reveal an immediate K^+ leak from the cells during this time interval, without a substantial alteration in the content of other ions. It is conceivable that K^+ loss without an immediate rehabilitation of its pool would very quickly lead to cell death as a result of K^+ replacement with other cations, such as Na^+.

Discussion

The treatment of cells by photosensitizers can lead to damage to different molecules, at various sites within the cell. The attempt to locate the exact damage site or the type of cellular activity most prone to be obstructed by singlet oxygen and lead to cell death is not trivial. It was indeed shown that photosensitized damage does occur to proteins, lipids and nucleic acids and that these reactions lead to a variety of cell-biological consequences [1,25,26].

It is obvious that the depolarization of the cell's resting potential and the free passage of ions and molecules through the cytoplasmic membrane are mutually interconnected. Specht and Rodgers employed a potentiometric fluorescent probe and have shown [27,28] that when mouse myeloma cells were photosensitized with zinc phthalocyanine sulfonate, plasma membrane depolarization was an early step in the in activation of the cells. Paardekooper et al. [29] showed that photosensitization of yeast cells by toluidine blue caused a drastic change in plasma membrane permeability. The dependence of the time-course of membrane depolarization on the dose of the sensitizer was also demonstrated [30]. Boegheim et al. [31] deduced from their measurements of mitochondrial activity that photosensitization lowers the plasma membrane potential. In contrast, Salet et al. [32] have shown that in photosensitization of isolated mitochondria the slow depolarization was probably not connected with the process of uncoupling of ATP synthesis. However, all these studies did not indicate whether the change in the electrical field on the membrane is the result, or whether it was the cause, of the lethal action of the photosensitization, and did not correlate the kinetics with possible mechanisms.

In this paper we attempted to answer the question whether the photo-induced death of the cells is caused purely by damage to their electrophysiological balance, by poring the encapsulating cytoplasmic membrane due to photodamage to the membrane's lipids. Alternately, is it plausible that the reported depolarization of the cell and the permeabilization of its membrane is actually the result of photoinduced damage to non-lipid systems, such as cellular, or membranal, protein systems and pumps?

This study was not carried out on one unique cell system but rather compared the rate of photosensitized reactions in different systems. In all the cases where the sensitizer was binding or partitioning to a microheterogeneous environment, such as BSA, liposomes or cells, the binding constants and the concentrations which were used ascertained that most of the sensitizer ($> 85\%$) was indeed bound. It does come out clear, however, that the relevance of pure photosensitized poration of an encapsulating lipid membrane is negligible under our photosensitizing conditions. This is evident from the almost non-existent leakage of the diffusion potential which is induced on liposomes and of the gradient of entrapped calcein in such vesicles. On the other hand, the photo-induced lethal damage to the bacterial and animal cells is achieved very efficiently with the same concentration of sensitizer and with the same laser's light intensity. These results are posted together in Fig. 2, which demonstrates the contrast bewteen cell-killing efficiency and depolarization of artificial membranes. We also found here that the photosensitization of simple protein models, such as DMI or BSA progresses with kinetics which are somewhat slower than the cellular action. A similar behavior is observed with membrane protein models: the retinal chromophore in bacteriorhodopsin and the tryptophan moiety in the same *S. aureus* cells.

An important piece of evidence in the chain of

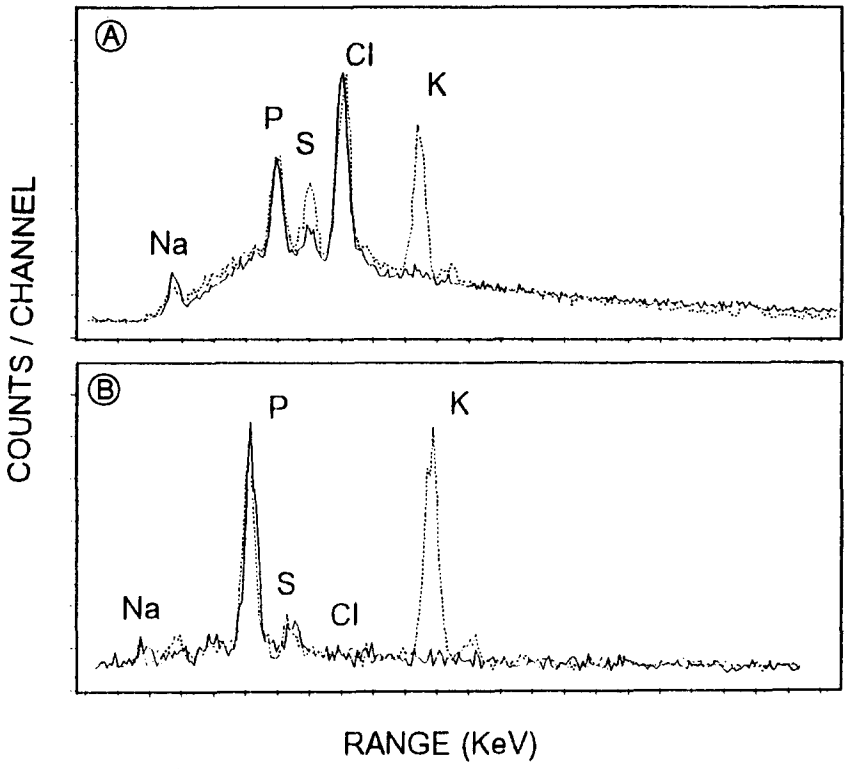

Fig. 5. The effect of photosensitization on K^+ content of erythroleukemic cells and *S. aureus* bacteria revealed by X-ray microanalysis. (A) X-ray spectra of FELC treated with HP (10 μM) and illuminated for 10 min; (B) X-ray spectra of *S. aureus* treated with HP (10 μM) and illuminated for 10 min. Dotted lines, control of unilluminated cells; full lines, light exposed cells. The cells were prepared and analyzed as described under Materials and Methods. The positions of the detected elements in the spectra is marked by the appropriate element's name.

events concerns K^+ leakage during photodynamic sensitization. There is an increased leakage of K^+ and Rb^+ during HPD treatment and light irradiation of L929 cells, similar to that described for red blood cells [33,34]. The increased passive permeability of cations ultimately leads to osmotic swelling and lysis. Rb^+ influx mediated by Na^+/K^+-ATPase was found to be very sensitive to the photodynamic action of hematoporphyrin [33,35,36]. The molecular mechanism of the photodynamically induced K^+ leakage is not yet totally elucidated [37]. Our present results (Fig. 5) show clearly that the loss of K^+ takes place in the short interval of 10 min of light exposure and explains the correlation between this leak and the death of the cells.

Based on these observations we conclude that the inactivation of cells does not result from a mechanical photo-poration of the membranes, which might lead to depolarization. It most probably arises from attack on protein sites which, among other effects, could also lead to the electrical depolarization of the cells.

Acknowledgements

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