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Volume III

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INTRODUCTION

Go outside and play in the sun. It's good for you.

My mother

The use of sunlight and drugs for the treatment of skin diseases has been documented for over 3400 years; for an even longer time, the reddening, blistering, and tanning effects of sunlight have probably been known. With the discovery of lasers a new dimension was added in the study and application of light in medical therapy. Ophthalmologists adopted the laser in the clinic as a photocoagulator for the treatment of detached retina, and the use of laser as a scalpel for noncontact, noninvasive, and even subcellular surgery is at an earlier state of acceptance. In addition to surgical uses, new, promising ideas are continuing to emerge. Thus, laser can be used to diagnose and treat malignant tumors using photoradiation therapy. This renewed interest, stimulated by the mutual interplay of both scientific and technological innovations, is characterized by a multidisciplinary approach involving physicists, chemists, biochemists, and physicians.

Our objective has been to collect in these three volumes the most up-to-date assessment of our understanding of light in medicine. Since *Photomedicine* was defined as an informative guide to practical applications rather than an esoteric study of medical discipline, the level of medical rigor was reasonably relaxed.

Given limitations on length, the chapters are not intended to be all embracing reviews of the field, but rather to present an overview of key ideas and directions with the objective of delineating the most promising and exciting problems. We hope that the text is sufficiently introductory to stimulate the curiosity and interest of a neophyte, and to simultaneously provide the specialist with a rather short, but current summary of the status of this field. Most important, we hope that the volumes will further highlight this rapidly developing science and spur current and new researchers and ideas.

Ehud Ben-Hur
Ionel Rosenthal

THE EDITORS

Ehud Ben-Hur, Ph.D., was born in Israel in 1940. After graduation from the Hebrew University of Jerusalem in 1965, he went on to study biochemistry at the Technion, Israel Institute of Technology at Haifa, where he obtained his M.Sc. and doctorate degrees. He then joined the Biology Department of Brookhaven National Laboratory as Research Associate where he completed postdoctoral work on the radiobiology of cultured mammalian cells under the auspices of Dr. M. M. Elkind. Upon returning to Israel in 1973, he first joined the Department of Cellular Biochemistry at the Hebrew University and then the Nuclear Research Center-Negev, in 1975, where he is currently engaged in studies of biological effects of ionizing and nonionizing radiations.

The main thrust of his research activity in the past was related to radiation-induced damage in DNA and its repair. During the last few years he has become interested in photodynamic therapy of cancer and is actively involved with Dr. I. Rosenthal in developing new and improved photosensitizers for this purpose.

Dr. Ben-Hur is affiliated with the Department of Radiation Biology, Colorado State University. He is also affiliated with Ben-Gurion University, Beer-Sheva, Israel, where he teaches photobiology. Dr. Ben-Hur has published over 80 papers in scientific journals, is a member of the American Society for Photobiology and the Radiation Research Societies of both the U.S. and Israel, and is on the Editorial Board of the *International Journal of Radiation Biology.*

Dr. Ben-Hur is married with two children and lives most of the time in Beer-Sheva.

Ionel Rosenthal, Ph.D., received his degree in Chemical Engineering from the Polytechnic Institute in Bucharest (Romania) and Ph.D. degree from the Freinberg Graduate School of the Weizmann Institute of Science, Rehovoth, Israel. Dr. Rosenthal has had a very colorful professional career which has included Plant Engineer at "Mahteshim" Chemical Co. and Senior Scientist at the Department of Organic Chemistry at the Weizmann Institute of Science and at the Department of Organic Chemistry, Nuclear Research Center-Negev. Currently he is Principal Scientist at the Department of Food Science, Agricultural Research Organization, Bet-Dagan, and Professor in the Department of Agricultural Biochemistry at the Faculty of Agriculture of the Hebrew University, Jerusalem.

His scientific interests in organic photobiochemistry and food chemistry (and its spin-off: cookery) have resulted in more than 100 research publications in these areas.

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Chapter 1

THE PHTHALOCYANINES: SENSITIZERS WITH POTENTIAL FOR PHOTODYNAMIC THERAPY OF CANCER

E. Ben-Hur, I. Rosenthal, S. G. Bown, and D. Phillips

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I. INTRODUCTION

Photodynamic inactivation of biological systems by exposure to certain dyes and visible light has been studied since the beginning of this century.[1] Oxygen is required for these photochemical reactions, which proceed either by direct interaction of the electronically excited dye molecule with a cellular target, followed by reaction of the transients formed with molecular oxygen (type I), or via interaction of the dye in its excited triplet state with oxygen and generation of active oxygen species (type II).[2] Clinical application of this process was prompted by the observation that malignant tumors can retain porphyrin derivatives for longer time than adjacent normal tissues. Subsequent exposure to light of proper wavelength can cause eradication of the tumor. This modality, termed photodynamic therapy, uses primarily hematoporphyrin derivative (HPD) as photosensitizer.[3] Although the main absorbtion band of HPD is around 400 nm, for therapy the dye is usually activated by red light ($\lambda = 630$ nm) where only a minor absorption peak exists, because of the increased transparency of tissues in the red.

HPD is a complex mixture of porphyrins of a somewhat variable composition derived from hematoporphyrin by treatment with a mixture of sulfuric acid and acetic acid (1:19).[4,5] This chemical reaction leads to formation of a large number of derivatives, dependent on the reaction conditions, which have been partially characterized as the dimethyl esters, dicarboxylic acids, and dehydro derivatives. The uncertainty of the active constituents in HPD and its chemical lability, may create difficulties in ensuring reproducibility in its use.

The potential advantage of phototherapy, of total and exclusive eradication of tumors without damage to surrounding tissues even in inoperable cases such as blockage of airways, justifies the search for alternative sensitizers. Ideally such a compound should (1) show a preferential retention/affinity for malignant tumors, (2) possess a very low toxicity, and (3) exhibit an efficient photodynamic effect activated by light of wavelength longer than 600 nm.

Metallophthalocyanines are porphyrin-like compounds (Figure 1) that absorb strongly in the red (the Q band, 600 to 700 nm, $\varepsilon \sim 10^5 \ell/\text{mol/cm}$). These dyes have important technological applications as catalysts, dyes, and pigments. There has also been a considerable interest in the photophysical properties of phthalocyanines since the first description of their photoconduction and semiconductor properties,[6] and their use as laser dyes.[7] The mechanistic photochemistry of several phthalocyanines has been investigated in recent years.[8-13]

The phthalocyanines can be easily synthesized and purified, and exhibit a very unusual chemical stability. Phthalocyanine derivatives are reported to be nontoxic. Thus, salts of copper phthalocyanine sulfonic acid are practically nontoxic to various species, from protozoa to dogs, in doses up to 100 mg/kg. Furthermore, this latter dye was reported to be retained preferentially in experimentally produced intracranial neoplasms in mice.[14] Similarly, uranyl tetrasulfonate phthalocyanine was shown to accumulate in brain tumors,[15] and (^{99}Tc) tetrasulfophthalocyanine concentrated to some extent in mammary adenocarcinoma in rats.[16] These observations make phthalocyanine derivates most promising photosensitizers for use in photodynamic therapy.[17] However, in spite of their structural resemblance to the biologically important porphyrins, the photobiology of phthalocyanines barely has been studied.

This chapter describes the photobiological activity of phthalocyanines in vitro and in vivo and indicates the potential for photodynamic therapy.

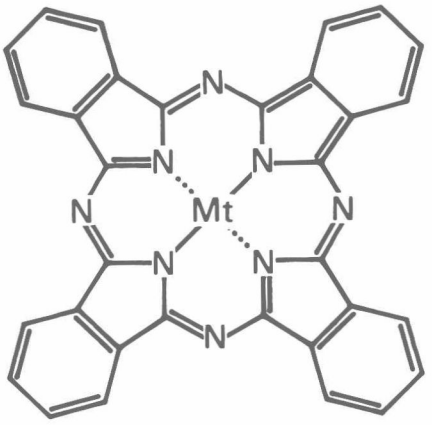

FIGURE 1. The molecular structure of metallophthalocyanine.

II. PHOTOBIOLOGY OF PHTHALOCYANINES IN VITRO

A. Photosensitized Killing of Cultured Cells

The initial observation that phthalocyanines can inactivate cultured mammalian cells following exposure to visible light was performed on Chinese hamster fibroblasts.[17] It was very early recognized that the metal atom complexed with the phthalocyanine ring was crucial for photobiological activity.[18] The screening assay of 11 different phthalocyanines indicated that aluminum phthalocyanine (AlPC) was most photobiologically active, followed by zinc derivative which was only one fifth as active under similar experimental conditions. Therefore, most of the experimental data were collected employing either AlPC or its water-soluble derivative containing sulfonic acid groups (AlPCS). Thus, exposure of Chinese hamster cells to white fluorescent light following an overnight incubation with AlPC causes exponential cell killing which is preceded by a pronounced shoulder (Figure 2). The shoulder on the survival curve was reduced, and the final slope became steeper when the concentration of AlPC in the growth medium prior to light exposure was increased. The cells became progressively more sensitive to light as a function of incubation time with the dye, reaching a maximum at about 3 hr for AlPC (Figure 3). The loss of sensitivity of the cells containing the dye, upon incubation in dye-free medium, is more rapid. This loss did not occur when incubation was carried out in medium or buffer devoid of blood serum. This is most probably due to the very low solubility of AlPC in water. In the growth medium, AlPC tightly binds to serum proteins,[19] which serve as a vehicle for its transport into and out of the cell.

The kinetics of photosensitization by AlPCS are much slower than those of AlPC, and saturation does not take place even after overnight incubation. The reverse process, loss of photosensitivity, is also slower with AlPCS as compared to AlPC.

The concentration dependence of photosensitivity (expressed as $1/F_{10}$, the reciprocal of the fluence required to reduce survival to 10%) induced by the two aluminum phthalocyanines is shown in Figure 4. While the response to both derivatives is linear over a wide range of concentrations, the slope of the AlPC curve is steeper and displaced upward, indicating a higher potency for the same concentration. While some of this difference is due to the fact that after 16-hr incubation, the effect of AlPCS has not yet reached a maximum (Figure 3), other factors might play a role. For example, their different solubilities could lead to AlPCS localization at intrinsically less-sensitive subcellular targets. The possibility that the two phthalocyanines have different photo-

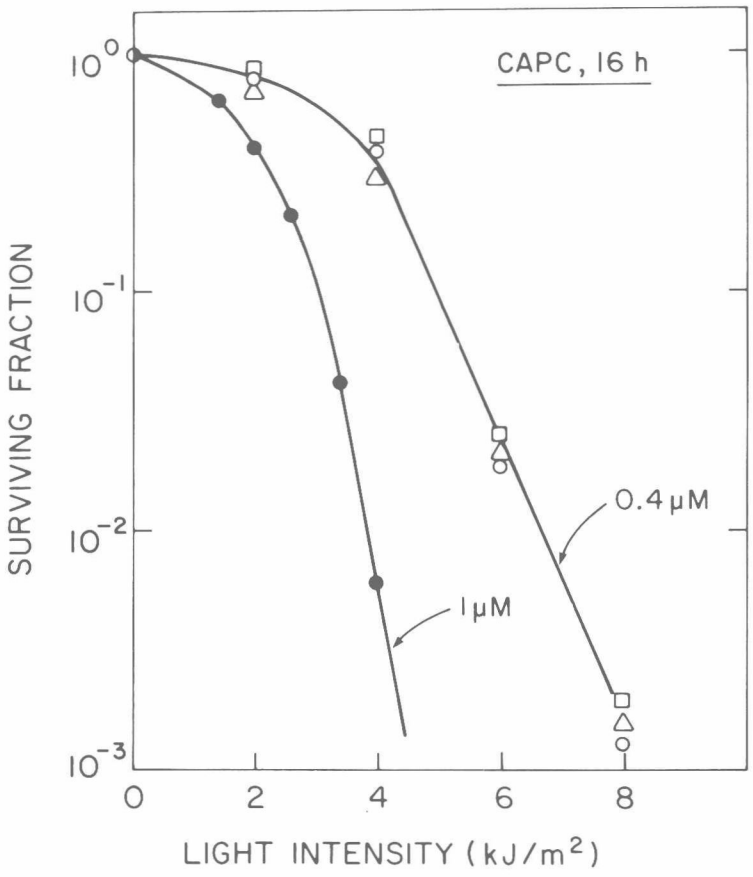

FIGURE 2. Survival of Chinese hamster cells exposed for 16 hr to 0.4 or 1 μM AlPC, as indicated, follwed by light exposure. Squares denote exposure to light in PBS containing 1 M glycerol, triangles are for PBS in D_2O, and circles are for PBS in H_2O.

reactivities is less likely since the photophysics of phthalocyanines is not expected to be affected by the substitution with sulfonic acid residues on the benzene rings.

B. Factors Affecting Photosensitization by Phthalocyanines

Cell physiology is an important factor that can determine the sensitivity of the cell to cytotoxic agents. The position of the cell in the cell cycle is crucial in this respect, and the response to UV and ionizing radiation displays a pronounced age structure (see Chapter 2). Thus, Chinese hamster cells are most sensitive to X-irradiation at the G_1/S border and are most resistant in late S phase. Conversely, the photosensitization by AlPC is equally efficient throughout the cell cycle, and the age-response function is virtually flat (Figure 5).

The external interference with the cellular processes operating, postphotoexcitation, to repair the photodamage, could result in enhanced sensitivity. In preliminary experiments, the production of DNA strand breakage was observed using the sensitive alkali elution technique. The yield of these breaks was about half of that produced by X-irradiation, at equisurvival doses. Such DNA breaks stimulate the synthesis of poly(ADP-ribose), which is involved in their repair. Inhibition of poly(ADP-ribose) polymerase, e.g., by 3-aminobenzamide (3-ABA), following exposure to ionizing ra-

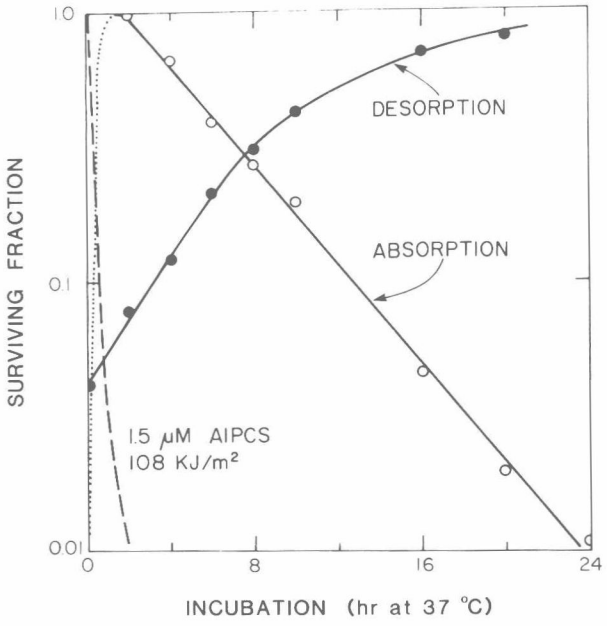

FIGURE 3. Kinetics of photosensitizaiton by phthalocyanines. Solid circles denote the development of photosensitivity as a function of time in the presence of 1.5 μM AlPCS followed by exposure to 108 kJ/m². The slashed curve is for 0.4 μM AlPC followed by 8 kJ/m². Open circles show the disappearance of photosensitivity when cells incubated for 16 hr with 1.5 μM AlPCS are rinsed and exposed to 108 kJ/m² after various incubation times in dye-free growth medium. The dotted curve is for 0.4 μM AlPC and 6 kJ/m².

diation, enhances cell killing.[20] In contrast, 3-ABA had no effect after exposure to AlPC and light (Figure 6).[21] This is consistent with the observation that the level of NAD^+, the precursor of poly(ADP-ribose) synthesis, is not depleted after photosensitization by AlPC. Presumably, the DNA damage produced is expressed as strand breaks only in the alkali conditions of the assay.

A more generalized treatment that inhibits repair processes operating on radiation damage is exposure to D_2O. Postirradiation incubation in D_2O medium enhances radiation response[22] and a similar effect occurs after AlPC photosensitization (Figure 6). It should be pointed out that the presence of D_2O during light exposure only, has no effect on the cell killing (Figure 2, see Section II.D for discussion). However, since in principle cells could repair sublethal damage during exposure that lasts for more than 1 hr at lower light fluence rate (see Chapter 2), the presence of D_2O under such conditions could increase the bioresponse by inhibiting repair processes.

C. Action Spectrum

Determination of action spectrum, i.e., the relative efficiency of various wavelengths in causing a specific bioeffect, is of fundamental importance in photobiology since it identifies the chromophore responsible for light absorption. In phototherapy, action spectrum indicates the optimal wavelength to be used for treatment. A dye laser pumped by a copper-vapor laser was employed for obtaining the action spectrum. The absorption spectrum of AlPC, in ethanolic solution, in aqueous solution after complexation with bovine serum albumin (BSA), or with yeast RNA, and the action spec-

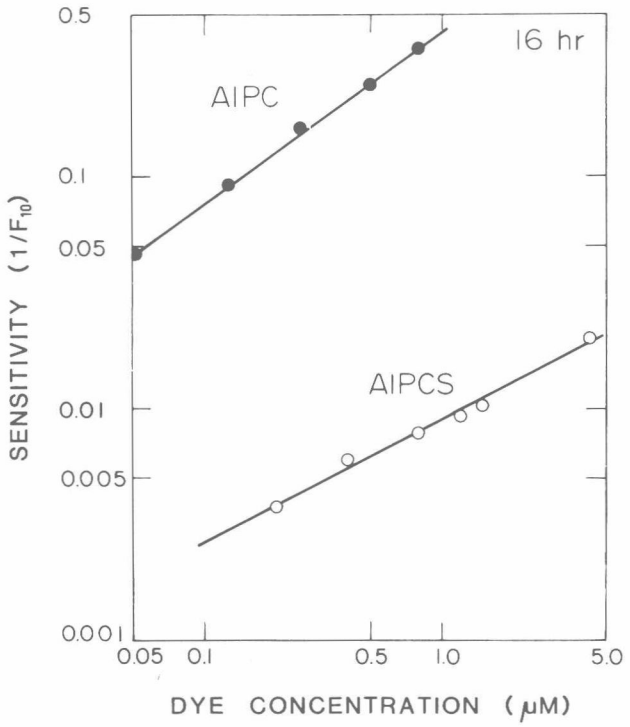

FIGURE 4. The dependence of Chinese hamster cell photosensitivity on phthalocyanine concentration. Cells were incubated for 16 hr with AlPC (•) or AlPCS (○) and the reciprocal of the fluence required to reduce survival to 10% ($1/F_{10}$) was plotted as a function of dye concentration.

trum are shown in Figure 7. It is evident that the absorption spectrum of AlPC is drastically modified after complexation with biological macromolecules. The binding constant is so high that the complex is not dissociated when run through a molecular sieve column of Sephadex G-25.[19] It is noteworthy that the action spectrum for Chinese hamster cell killing follows none of the absorption spectra of the complexes of AlPC. This would tend to rule out proteins and RNA as the cellular targets for AlPC action in the cell. Since no dye binding to DNA could be observed, and because of the dye hydrophobicity, a most likely target is the lipid constituents of cellular membranes.

Unlike white fluorescent light, survival curves resulting from laser exposure were essentially exponential. The fluence required to kill 90% of the cells was approximately tenfold lower using 680-nm laser light than a fluorescent lamp. While the average fluence rate was similar in both cases, the laser in contradistinction to fluorescent light consisted of 4000 pulses per second. Thus, the actual power density during the pulse was much higher than during exposure to fluorescent light and could drastically modify the photophysics of the sensitizer. This could be the reason for the disappearance of the shoulder from the survival curve. At any rate, the action spectrum suggests that wavelengths about 680 nm should be employed for maximum photoefficiency in sensitization reaction with AlPC.

D. Mechanism of Photosensitizing Inactivation

The first question that arises concerning the mechanism of phthalocyanine photosensitization is the involvement of molecular oxygen. Figure 8 shows that oxygen is defin-