Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 79-84

EVALUATION OF CHEMICALLY-INDUCED PHOTOTOXICITY TO AQUATIC ORGANISM USING PARAMECIUM AS A MODEL

Prakash C. Joshi and R.B. Misra

Phototoxicology Laboratory, Gheru Campus
Industrial Toxicology Research Centre
Post Box 80, Mahatma Gandhi Marg
Lucknow-226001, India

Received July 10, 1986

Phototoxicity evaluation using Paramecium aurelia as a model revealed that 4 out of 21 pesticides produced lethal toxicity to cells. Four commonly used synthetic dyes (bromophenol blue, rose bangal, benzanthrone and methylene blue) also exhibited toxicity. Well known phototoxic agents like hematoporphyrin, riboflavin, and anthracene produced positive phototoxic response. Psoralen, a DNA cross-linking agent, also produced phototoxicity to the cells. The results clearly demonstrate that the synergistic action of chemical agents and sunlight produce lethal effects to aquatic organism.

Continuous effluence of industrial, agrochemicals, and synthetic or natural chemical agents into water leading to a series of changes in the morphology of aquatic organism has caused a deep concern in recent years. Some of the environmental pollutants, such as petroleum products, dyes, food additives, pesticides, drugs, etc., are known to induce enhanced toxicity in the presence of natural or artificially-produced light (1-6). Most of these photosensitized reactions are referred to as photodynamic reactions and require the participation of O_2 (7,8). In other photoreactions, the direct combination of a photosensitized molecule with a biological substrate is more apparent (9). Both the O_2-dependent and O_2-independent reactions contribute to the oxidative degradation of target molecules of cellular systems (7,8,10-13). Thus, aquatic organisms, mammalian cells, and plants are all vulnerable to chemical phototoxicity. In the present study, we selected 29 chemicals (see table) from various categories of organic compounds. The majority of these are pesticides and dyes. HPD, riboflavin, anthracene, and PSO were used as positive controls. The choice of Paramecium as a

ABBREVATIONS: HPD, hematoporphyrin; PSO, psoralen; $^1O_2$, singlet oxygen; $O_2^{\cdot}$, superoxide radical.

0006-291X/86 $1.50

model for assessing phototoxic potential was based upon its well characterized eukariotic nature of the organism, world-wide abundance, and convenient maintenance in culture under laboratory conditions and natural light.

MATERIALS AND METHODS

Paramecium aurelia was collected from nature near our laboratories. Cells were grown in cerophyl infusion inoculated with Klebsiella as reported earlier (14). Twenty one pesticides, four dyes, and other chemicals such as anthracene, riboflavin, and some known photosensitizers were examined for phototoxicity. They represented a broad spectrum of various categories of potentially hazardous synthetic and naturally occurring chemical agents. Most of the test compounds (e.g., pesticides, dyes, etc.) were selected because of their likely contamination with the natural water reservoirs and potential health hazard to aquatic organism while the remainder [e.g., HPD (15), riboflavin (16), and PSO (17)] were selected on the basis of their well established photosensitization characteristics. Test chemicals were obtained principally from Sigma Chemical Company. Benzanthrone was obtained from Aldrich Chemical Company. Pesticides were generously provided by the US Environmental Protection Agency, Pesticides and Industrial Chemical Repository, Research Triangle Park, NC, USA. Prior to use, their purity was checked by TLC and HPLC. Their UV and visble spectra were recorded on a Pye-Unicame-800 Spectrophotometer. Stock and irradiation solutions were prepared in Dryl's medium (18). Both control and experimental animals were washed in Dryl's medium prior to irradiation. One hundred cells (in 10 ml Dryl's medium) were taken in a petri dish (6-cm diameter) containing 0 to 5 µg/ml test compound. Irradiation was done on sunny days preferably between 9:30 to 11:30 a.m. (temperature maintained at 20° ± 2° C). The irradiance of sunlight was measured by an IL730A UV Actinic Radiometer (International Light Inc., Newburyport, MA, USA), and the recorded mean fluence was 1.2×10^{-2} mW/mm^2 in the UVA (320 - 400 nm) range and 1×10^{-4} mW/mm^2 in the UVB (290 - 320 nm) range. The motility changes and mortality rate were examined under a Sterio Microscope (Wild-M_3, Heerburg, Switzerland).

RESULTS

Control experiments revealed no toxicity or lethality to Paramecium with the test chemicals (20 - 25 µg) when the organisms were kept in the dark at room temperature for up to six hours. Preirradiated chemicals (0.5 to 25 µg/ml) under sunlight exposure (90 - 120 minutes) did not affect the cells kept in the dark indicating photodegradation products of the test compounds were not responsible for cellular damage. Pretreatment of cells with chemicals (25 µg/ml) for a period up to six hours at room temperature in the dark, and subsequent removal of the test chemical from cells by repeated washings was also found to have no lethal or toxic effect to the cells when they were exposed to sunlight for 90 minutes. Only the synergistic action of solar radiation in the presence of some of the chemical agents produced toxicity to cells as evidenced by their loss of motility and viability. The nonviable cells submerged to the bottom of the petri dish. The degree of cell mortality (measured by counting the number of living and motile

cells under a microscope) depended upon the concentration of the chemical agent and sunlight exposure dose. All the four dyes tested (bromophenol blue, rose bengal, benzanthrone, and methylene blue) produced 40% to 100% mortality within 90 minutes of sunlight exposure. Most dyes, in general, show absorption peaks in the visible spectral range (400 - 700 nm); therefore, visible radiation appears to play a significant role in the dye induced phototoxicity to Paramecium. The majority of pesticides, however, did not show enhanced phototoxicity to cells. Dicofol (1.0 μg/ml) and endosulfan (1.0 μg/ml) were toxic and caused mortality to cells in the dark without exposure to sunlight. Cells survived in sunlight (90 minutes) at a low concentration (1.0 μg/ml) using the following pesticides: dithan-78, dithan-45, dodine, syllet, copper oxychloride, decis, 2-4 D, BHC, endocel, ethion, lindane, and DDT; however, they died at a high concentration (5 μg/ml) using the same pesticides even without sunlight exposure. Malathion and dimethoate did not produce toxicity to cells up to 5 μg/ml level with or without sunlight. Only four pesticides produced phototoxicity to Paramecium by their synergistic action with sunlight in the following order: moncozeb > karathan > thanite > zineb (Table 1).

DISCUSSION

Analysis of the results summarized in the table revealed the cells exposed to solar radiation at various time intervals were killed only in the presence of a few compounds. HPD, riboflavin, anthracene, and benzanthrone produced maximum phototoxicity along with the industrial dyes. The lethal photosensitization potential of HPD is well known and is related to its ability to generate $^1O_2$, a reactive species which damages cell membranes (19). The ability of riboflavin to induce $^1O_2$ and $O_2^{\cdot-}$ production and DNA damage has been recently studied in our laboratory (16). The photochemically-induced toxicity of anthracene to juvenile sunfish has also been reported recently (5). PSO, a naturally occurring furocoumarin and a photochemotherapeutic drug, was used as a positive control in view of its well known phototoxic properties (20). PSO photoconjugates covalently with DNA to form interstrand cross-links and also generates reactive O_2 species ($^1O_2$ and $O_2^{\cdot-}$) to induce cell death (9).

TABLE 1: SURVIVAL OF PARAMECIUM AGAINST SUNLIGHT-INDUCED PHOTOTOXIC EFFECTS OF VARIOUS CHEMICAL AGENTS

No. & Compound	Concentration (µg/ml)	Percent Survival of Cells					
		Unexposed Cells at 90 minutes*	Sunlight Exposure (Minutes)				
			15	30	45	60	90
A - Pesticides							
1. Arelone	1.0	100	100	100	100	100	100
	5.0	10	100	75	37	15	10
2. BHC	1.0	100	100	100	100	100	100
	5.0	50	100	100	84	72	50
3. Copper oxy Chloride	1.0	100	100	100	100	100	100
	5.0	0	100	62	30	0	0
4. 2-4-D	1.0	100	100	100	100	100	100
	5.0	25	100	95	87	38	25
5. DDT	1.0	100	100	100	100	100	100
	5.0	55	100	100	100	75	55
6. Decis	1.0	100	100	100	100	100	100
	5.0	20	100	83	70	55	20
7. Dicofal	1.0	0	100	90	0	0	0
8. Dimethoate	5.0	100	100	100	100	100	100
9. Dithan-45	1.0	100	100	100	100	100	100
	5.0	0	26	5	0	0	0
10. Dithan-78	1.0	100	100	100	100	100	100
	5.0	0	10	0	0	0	0
11. Dodine	1.0	100	100	100	100	100	100
	5.0	0	28	17	10	0	0
12. Endocel	1.0	100	100	100	100	100	100
	5.0	30	85	60	44	37	25
13. Endosulfan	1.0	0	100	97	78	30	0
14. Ethion	1.0	100	100	100	100	100	100
	5.0	20	51	40	35	21	17
15. Karathan	1.0	100	100	100	100	100	100
	5.0	95	100	30	10	0	0
16. Lindane	1.0	100	100	100	100	100	100
	5.0	28	87	75	43	42	30
17. Malathion	5.0	100	100	100	100	100	100
18. Moncozeb	1.0	100	100	100	100	100	100
	5.0	98	95	11	5	0	0
19. Syllet	1.0	100	100	100	100	100	100
	5.0	0	35	27	15	13	3
20. Thanite	0.1	100	100	100	100	100	100
	1.0	100	100	100	87	80	68
21. Zineb	0.1	100	100	100	100	100	100
	1.0	100	100	100	100	97	90
B - Dyes							
1. Benzanthrone	0.1	100	100	97	71	36	7
	1.0	100	0	0	0	0	0
2. Bromophenol blue	0.1	100	100	100	100	100	100
	1.0	100	100	97	75	71	60
3. Methylene blue	0.1	100	100	100	97	90	83
	1.0	100	80	70	29	25	17
4. Rose bengal	0.1	100	100	100	97	93	76
	1.0	100	85	63	35	27	0
C - Others							
1. Hematoporphyrin	0.1	100	100	100	100	100	100
	1.0	100	91	85	65	61	35
2. Riboflavin	0.1	100	100	100	100	100	100
	1.0	100	90	80	71	52	40
3. Psoralen	0.1	100	100	100	94	90	80
	1.0	100	75	60	30	21	4
4. Anthracene	0.1	100	90	27	0	0	0
	1.0	100	0	0	0	0	0

* (1) These cells were observed after 90 minutes in the dark in the presence of test compound.
(2) In some cases (Group A1-7, A9-14, and A16-19), when partial to complete cell lethality was observed even without sunlight exposure, the overall effect may not be considered as phototoxic.

The mode of phototoxic action of certain chemicals is believed to be primarily due to their photoactivation to energy-rich electronically excited states known as singlet excited and triplet excited states (6). These photoexcited molecule may

exert its phototoxic property by various mechanisms. With HPD, riboflavin, PSO, and anthracene, the following two types of reaction mechanisms appear to be primarily responsible for their photosensitization potential: (a) Type I or O_2 independent mechanism in which a photoexcited molecule reacts convalently with a substrate, and (b) Type II or O_2-dependent reaction when the photoexcited molecule in its metastable triplet state transfers the absorbed energy to molecular O_2 giving rise to the formation of $^1O_2$ and $O_2^{\cdot-}$. The radicals produced are responsible for biological damage (photodynamic reactions). HPD and riboflavin, respectively, are known to produce large quantities of $^1O_2$ and $O_2^{\cdot-}$ via O_2-dependent photodynamic reaction mechanisms (16,17). Several dyes are also known for their photodynamic oxidation characteristics (7,21). The reactive O_2 species are now believed to play a signficant role in skin photosensitization reactions. It is interesting to note that zineb, an agricultural fungicide which has been found to be phototoxic to Paramecium in this study, is also known for its skin and mucus membrane irritation reactions (22).

The results of this study demonstrate that industrial pollutants which undergo photosensitization may produce toxicity to aquatic organism, e.g., Paramecium in the present case. Higher aquatic organisms rely on the lower organisms for their food and survival. With the increase in toxicity due to photochemical reactions of environmental pollutants in water, the entire chain of ecological events may become disrupted faster than the anticipated rate. One question may be raised as to what level in water are the aquatic organism vulnerable to phototoxicity? It will obviously depend upon the level of sunlight penetration. In clear water, visible light (400 - 760 nm) penetrates with decreasing intensity up to three meters. In polluted and turbid water, the depth of penetratino may be less. Another significant set up for the occurrance of phototoxic reactions is that the photosensitizing molecules (or cells) may not be absorbed by cells but should be present in the vicinity of chemical agents. Photoexcited molecules normally have a short half-live (of the order of 10^{-8} second). Electronically excited $^1O_2$ has a relatively long lifetime (10^{-3} - 10^{-2} seconds) and can diffuse through the cell membranes resulting in membrane and nuclear damage.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. V. Sundararaman for generously extending the facilities of his laboratory and stimulating discussion on Paramecium and to Dr. Madhu A. Pathak of Harvard Medical School, Boston, Massachusetts, USA, for his assistance in the revision of this manuscript.

REFERENCES

1. Scheier, A., and Gominger, D. (1976) Bull. Environ. Contam. Toxicol. 16, 595-603.
2. Gesser, H.D., Wildman, T.A., and Tewari, Y.B. (1977) Env. Sci. Technol. 11, 605-608.
3. Larson, R.A., and Hunt, L.L. (1978) Photochem. Photobiol. 28, 553-555.
4. Calkins, J. (1982) The Role of Solar Ultraviolet Radiations in Marine Ecosystem, Plenum Pub. Co., New York.
5. Oris, J.T., and Giesy J.P. (1985) Aquatic Toxicol. 6, 133-146.
6. Megaw, J.M., and Drake, L.M. (1985) J. Toxicol. Cut & Ocular Toxicol. 4, 153-183.
7. Foote, C.S., (1976) Free Radicals in Biology (Pryer, W.A., ed.), Vol. 2, pp. 85-133, Academic Press, New York.
8. Ito, T., (1978) Photochem. Photobiol. 28, 493-508.
9. Pathak, M.A., and Joshi, P.C. (1983) J. Invest. Dermatol. 80, 66s-74s.
10. Clagett, D.C., and Galen, T.J. (1971) Arch. Biochem. Biophys. 146, 196-201.
11. Krinsky, N.I. (1979) Singlet Oxygen (Wasserman, H.H. and Murray, R.W., eds.), pp. 597-667, Academic Press, New York.
12. Floyd, R.A. (1982) Free Radicals and Cancer, Marcel Dekker. A.G., Switzerland.
13. Van Steveninck J., Dubbelman, T.M.A.R, and Verweij, H. (1983) Adv. Exp. Med. Biol. 160, 227-240.
14. Sonnerborn, J.S. (1950) J. Exp. Zool. 113, 87-148.
15. Boye, E., and Moan, J. (1980) Photochem. Photobiol. 31, 223-228.
16. Joshi, P.C. (1985) Toxicol. Lett. 26, 211-217.
17. Pathak, M.A., and Joshi, P.C. (1984) Biochemica Biophysica Acta 798, 115-126.
18. Dryl, S., (1965) Exp. Cell Research 37, 569-581.
19. Moan, J., Sandberg, S., Christonsen T., and Elender, S., (1983) Adv. Exp. Med. Biol. 160, 165-179.
20. Gabriel, K.L., Forbes, P.D., and Davies, R.E. (1985) J. Toxicol. Cut. & Ocular Toxicol. 4, 185-191.