Chapter 8

Physiological Effects of Photodynamic Action: Special Reference to Insects

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Results of studies involving photodynamic action and its effect on the physiology of organisms, especially insects, are summarized and discussed. With regards to insects, few studies on the photooxidative reactions were conducted until the early to mid-seventies. Since that time, there has been a renewed effort to determine how photodynamically active compounds affect insect physiology. This chapter is a review of findings on this phenomenon as it affects the organism at (1) the subcellular level, (2) the cellular level, (3) the systems level, and (4) aspects of photodynamic action as it affects development and reproduction.

The physiological effects of photodynamic action have been studied in a variety of organisms in the last eight decades. However, only four original papers from 1900 to 1970 reported on insects as the experimental entity. While some earlier studies (1970-1975) designed to evaluate the phototoxic effects of photodynamically active substances included observations on physiological effects, this subject received little attention from researchers until later in the decade. Beginning in the mid-seventies, and to the present time, several studies have been conducted in renewed efforts to elucidate the effects that are associated with dye-sensitized photooxidative reactions in insects. These studies have added greatly to our knowledge of how photodynamically active substances affect insects (and other organisms) from the cellular level to the systems level.

The phenomenon of photodynamic action has been the subject of several reviews. Some of the works cited in those reviews have also been cited in this chapter where relevant. These reviews are not all inclusive and deal with aspects of photodynamism other than physiological ramifications in living organisms. The following are general reviews of photodynamic effects on cells and multicellular organisms that may be of interest to the reader:

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Blum (1), Errera (2), Clare (3), Fowlks (4), Santamaria (5-6), Santamaria and Pinto (7), Spikes and Ghiron (8), Simon (9), Spikes and Straight (10), Bourdon and Schnurizer (11), Spikes (12), Spikes and Livingston (13), Pooler and Valenzeno (14), and Robinson (15).

The purpose of this review is to summarize and discuss findings on the physiological effects of the photodynamic action at (I) the subcellular level, (II) the cellular level, (III) the systems level, and (IV) aspects of the photodynamic action as it affects development and reproduction in insects.

Effects at the Subcellular Level

Many studies on vertebrates and invertebrates have shown that "almost no cell process or structure is immune from ... photosensitized modification under the right conditions" (14). Sensitized molecules are able to oxidize a wide range of cellular components including intermediates, proteins, and membranes.

Pooler and Valenzeno (14) cite several examples of photochemical damage and modifications occurring to intracellular components by photosensitizing agents. Once permeability of the cell membrane has been altered, cellular function can be greatly modified. Cande et al. (16) reported that lysed cells of kangaroo rat kidney are permeable to small molecules such as erythrosin B. They observed that holes were present in the plasma membrane and the mitochrondia were swollen and distorted; other membrane-bound organelles were not noticeably altered. Haga and Spikes (17) also reported swelling of isolated rat liver mitochondria sensitized with eosin Y and methylene blue. On the basis of certain metabolic measurements, they concluded that the observed effects of these phototoxins suggested that the swelling is caused by inhibition of enzymatic activities in the electron transport system and by the uncoupling of phosphorylation from respiration. Hilf et al. (18) have also proposed that mitochrondia may be a critical subcellular site of photomodification.

Loss of cellular potassium has been reported and, in turn, protein synthesis and cell membrane potentials are affected. Lysosomal damage by photosensitizers can result in secondary cellular damage by altering the fine structure of mitochondria and endoplasmic reticulum. Unsaturated lipids, nucleic acids, DNA, and RNA may be modified photochemically. Proteins and certain of their amino acid side chains are susceptable to photosensitized attack.

Palm (19) noted that basic dyes like methylene blue and neutral red can produce granules in the cytoplasm of insect cells with neutral red granules often representative of vacuoles. Other workers (see Pooler & Valenzeno (14)) have reported cytoplasmic vacuolization or blebs appearing in photosensitized cells.

Only a limited number of studies of photodynamic action in insects at the subcellular level have been conducted. Carpenter et al. (20) studied the synergistic effect of fluorescein on rose bengal in the presence of a purified enzyme. They showed that fluorescein enhanced the photodynamic activity of rose bengal in

the inhibition of glyceraldehyde-3-phosphate dehydrogenase of mosquito larvae (Aedes triseriatus (Say)).

Fondren and Heitz (21) observed LT_{50} and tissue levels of dyes and suggested that the face fly (Musca autumnalis DeGeer) is susceptable to toxic intracellular reactions of certain sensitized xanthene dyes. Fondren et al. (22) conducted similar studies on the house fly (Musca domestica L.) where "relative toxicities were described by means of rate constants of photooxidation calculated for (6 xanthene dyes) which included both the LT_{50} and the tissue dye level."

Callaham et al. (23) reported that dye-sensitized photooxidation of the acetylcholinesterase in whole-head homogenates of the imported fire ant (Solenopsis richteri (Forel)) could be induced in the presence of several xanthene dyes. Lactic dehydrogenase and acetylcholinesterase of the boll weevil (Anthonomus grandis grandis Boheman) were inactivated by dye-sensitized photooxidation mediated by substituted xanthenes (24).

There is considerable evidence that many photodynamically active compounds are mutagenic. It has been suggested that all mutagenic chemicals react with the protein part of the gene molecule rather than with the nucleic acid (25). Clark (25) studied the mutagenic activity of several dyes in Drosophila melanogaster Meigen and suggested that the dye molecule may react with either the nucleic acid or protein moiety of the gene molecule. Evidence from the study suggested that pyronin (a thiazine dye) produces a genetic effect by combining directly with nucleic acid. He also suggested that the mutagenic activity of a dye is related to its affinity for unpolymerized nucleic acid. In this study, he reported that neutral red was capable of producing 0.33% lethals and that rhodamine appears to be definitely although weakly mutagenic causing 0.27% lethals. The mutagenicity of acridine compounds in different biological systems was reviewed by Nasim and Brychcy (26). Acriflavine and acridine orange were reported to increase sex-linked and second chromosome recessive lethal mutations in both males and females of D. melanagaster. In the silkworm, Bombyx mori (L.), acridine orange produced mutagenic effects in egg-color loci in female but not male pupae. However, mutagenic effects were observed with parental chromosomes in mitotic cleavage nuclei.

Bianchi et al. (27) studied the effects of methylene blue on fixed eukariotic chromosomes of the mosquito Culiseta longiareolata under aerobic conditions with visible light irradiation. They found that diluted solutions of the dye dramatically altered chromosomal structure. Results of this study suggested that electronically excited O_2, a specific product of the interaction among visible light, methylene blue, and O_2, may be responsible for chromosomal DNA alteration. Similar conclusions were drawn by Gruener and Lockwood (28) in a study on photodynamic mutagenicity of rose bengal in Chinese hamster embryo cells. Commercial rhodamine 6G and rhodamine B have been shown to induce reversion mutations in Salmonella and single-strand breaks in Chinese hamster ovary cells (29). However, anochlor 1254-induced rat liver homogenate (S9) is required for production of genetic activity by these dyes. The photomutagenic effects of chlorpromazine in

Salmonella and Chinese hamster ovary cells were studied by Ben-Hur et al. (30). They found a pH related effect which facilitated binding of this phototoxin to DNA, RNA, and proteins within the cells that enhanced phototoxicity and mutagenicity. Plant-derived furanoquinolines and certain tryptophan-derived alkaloids were shown to inhibit mitosis and to cause chromosomal aberrations in microorganisms (bacteria, fungi) and in Chinese hamster ovary cells (31).

Effects at the Cellular Level

Besides some of the subtle to the more obvious, dramatic effects photodynamically active compounds have on cellular components, several photosensitizers can cause complete destruction of cells. Destruction most likely begins with the altered permeability of the cell membrane which in turn allows intracellular pertubations to occur which ultimately results in the demise of the entire cell (e.g. release of lysosomal material to the cytoplasm with subsequent cell lysing and complete destruction). Additionally, the cellular site of damage and/or mode of damage is apparently dependent on the sensitizer and its localization.

There are a number of reports on the effects of photosensitizers on whole cells of organisms other than insects (see review articles). Blum (1), in his review of photodynamic action, cites findings by various workers on effects of phototoxins on mammalian blood cells. Under light conditions, erythrocytes were hemolyzed and reduced in number by several xanthene dyes. Hemolysis also was observed in the absence of irradiation by high concentrations of rose bengal. More complex changes have been noted in total leucocyte counts under *in vitro* irradiation; numbers may gradually increase from a normal level to a condition of leucocytosis followed by leucopenia.

The review by Pooler and Valenzeno (14) discusses photodynamic inactivation of erythrocytes. Membrane photomodification has been extensively studied in these cells. In the presence of an appropriate sensitizer and light, there is progressive cell swelling eventually culminating in lysis with release of cell contents; the swelling and lysis are said to be of a colloid osmotic nature. No similar observations have been made on insect hemocytes. Given the morphological and presumed functional diversities of hemocytes found free in the hemolymph and associated with hemopoietic tissue or organs, some interesting observations await researchers who undertake studies of the effects photodynamic action have on these cells in insects.

In the only known study of the effects of photosensitizers on insect hemocytes, Weaver et al. (32) showed that erythrosine B significantly affected the aggregate of hemocytes in the American cockroach (Periplaneta americana (L.)). Light-exposed, dye-injected roaches showed diminishing numbers of hemocytes at dosages from 0.068 and 0.244 mg of dye/g of body weight, resulting in reductions of 11 and 40%, respectively. Light-exposed, dye-fed roaches also tended to have fewer hemocytes than untreated controls. Roaches held in darkness and either dye-fed or dye-

injected tended to have higher levels of hemocytes in all cases except at the highest injected dose of 0.244 mg; at this dose, there was a significant reduction of nearly 25%. The reason(s) for increased levels in roaches not irradiated are not known and similar phenomena have not been noted in mammalian systems. It was suggested that the dye alone may have affected the adhesion of cells (e.g. cystocytes) to tissue, thus driving normally noncirculating hemocytes into circulation. This study did not determine if lysis was the cause of the decreased number of hemocytes observed. The possibility was mentioned that the dye/light treatment may have caused an increase in the number of adhering hemocytes rather than causing lysis. When considering the aggregate of cells and the changes noted, the dye apparently induced an initial condition of leucocytosis that progressed to a state of leucopenia with increased dosage upon irradiation; similar observations have been noted in mammalian systems.

One of the more common effects of phototoxins on insect cells that has been observed is that of lysing. Yoho (33) presented evidence of lysing of midgut epithelial cells in the house fly. Schildmacher (34) (as cited by Respicio and Heitz(35)) observed considerable destruction of the midgut wall in mosquito larvae treated with acridine red. Carpenter and Heitz (36), in their study on latent toxicity of rose bengal on larvae of Culex pipiens quinquefasciatus Say observed that the gut tract in treated larvae appeared destroyed.

The coagulation of insect hemolymph appears to be affected by photosensitizers. In their study on hemocytes, Weaver et al. (32) observed that gelation of the plasma was often adversely affected in erythrosin B-treated roaches (unpublished results). Treated roaches bled more freely with less coagulation than did untreated controls. This could be a direct result of diminished numbers of cystocytes in hemolymph; cystocytes have been shown to be associated with coagulability of the blood in Periplaneta.

Effects at the Systems Level

Influence on Components of Body Fluids. Biochemical changes in insects induced by phototoxins have been studied by only a few workers. Broome et al. (37) conducted in vivo studies of the biochemical changes associated with the dark reaction of dietary rose bengal in the boll weevil. Inclusion of rose bengal in the diet of newly-emerged boll weevils for four days decreased levels of total lipids (90%) and total proteins (41%) when compared to controls. The total amino acid pool increased 3%; lysine, glycine, tyrosine, histidine, arginine and proline increased, whereas the remaining amino acids either decreased or remained the same. In a related paper, Callaham et al. (38), using dietary rose bengal, studied similar biochemical parameters in the adult boll weevil through five consecutive days post-emergence. In the controls, protein levels nearly doubled at two days post-emergence then remained fairly constant from 2-5 days; treated adults maintained the same level through the five day period. Amino acid pool sizes at five days for the controls and treated adults showed decreases of 14 and 20%, respectively, when compared to controls

at day 0. Enzyme activity of lactic dehydrogenase and acetylcholinesterase also showed a decrease in treated weevils. In both studies, the authors suggest that rose bengal can cause a lethal energy stress on the organism.

Hemolymph proteins of erythrosin B-treated adult American cockroaches have been studied by polyacrylamide disc electrophoresis (Weaver, J. E., West Virginia University at Morgantown, unpublished data). Protein patterns in dye-sensitized roaches were altered in position and numbers and the mean concentration of protein within specific bands was significantly different from untreated roaches. There were distinct differences between sexes and irradiated versus dark-treated roaches.

Influence on Body Fluids. The xanthene dyes, rose bengal and erythrosin B have been shown to cause volumetric changes in the hemolymph and crop contents of dye-sensitized American and oriental cockroaches (Blatta orientalis L.) (39). Dietary or injected dye were both effective in producing significant changes in hemolymph and crop volumes but changes were more dramatic, especially in crop volumes, in injected roaches. In irradiated roaches, hemolymph volumes progressively decreased and crop volumes increased over time up to 63 minutes. It was suggested in this study that cell membrane permeability may have been affected thereby creating a differential in osmotic pressure which allowed hemocoel fluids to pass into the alimentary canal.

Changes in the specific gravity of hemolymph in the American cockroach have been observed after photosensitization with erythrosin B. Irradiated, sensitized roaches showed increases in specific gravity of 0.44 and 0.81% after 30 and 60 minutes of light response, respectively (Amrine, J. W. Jr., West Virginia University at Morgantown, unpublished data). These changes could be related to a loss of water through the Malpighian tubules into the alimentary canal with a concurrent impairment (failure) of the water retrieval system in the lower Malpighian tubules and rectal membrane precipitated by the photodynamic action.

Influence on the Nervous System. Studies on a number of organisms have shown that excitable cells are susceptable to photomodification. Normal impulse propagation and subsequent events triggered by the impulse may become blocked or the impulse distorted in complex ways in sensitized cells depending on the light dose. Pooler and Valenzeno (14) provide a good review of photodynamic inactivation of excitable cells in nerve axons, skeletal muscle, cardiac and smooth muscle in various organisms other than insects (see also preceeding chapter). Kondo and Kasai (40) studied the photoinactivation of sarcoplasmic reticulum vesicle membranes of rabbit by several xanthene dyes (erythrosin B, eosin Y, rhodamine B, methylene blue, rose bengal). They observed that some regular relationships exist between the molecular structures of xanthene dyes and the inactivation of these excitable cells. Food, drug and cosmetic dyes of the xanthene type have been shown under dark conditions to act in a dose-dependent manner when applied to isolated molluscan ganglia; these dyes alter the potassium permeability of the membrane

thereby increasing the resting membrane potential and conductance of the neurons (41). A later study by Augustine and Leviton (42) showed that light intensity was also a factor in the degree of activity observed with erythrosin B on the presynaptic effect of this xanthene dye at the frog neuromuscular junction. In a study on the synaptic connectivity between auditory interneurons of the cricket, Gryllus bimaculatus DeGeer, Selverston et al. (43) used the intracellular dye, lucifer yellow, to selectively photoinactivate single neurons in prothoracic ganglion.

Various behaviorial responses observed in insects subjected to photoreactive substances strongly suggest that xanthene dyes, especially, can seriously affect the nervous system. Yoho et al. (44) observed that sensitized house flies after a dark period were initially very active when first exposed to light. Periods of hyperactivity, characterized by sporadic bursts of flying and prolonged antennal and wing-cleaning movements, were followed by periods of quiescence. Prolonged movement of the labellum was associated with regurgitation and defecation. Locomotory movements became uncoordinated as flies lost functional control of legs; simultaneously, ovipositors of females were often osberved in an extended position. Flies became progressively uncoordinated, often falling onto their side or dorsum. Many flies died with legs folded ventrally over the thorax; others died in a normal upright position. Broome et al. (45) noted that imported fire ants sensitized with rose bengal exhibited increased irritability, increased antennal grooming, loss of locomotory coordination, followed by a tetanic paralysis prior to death. The ants quite often assumed "a contorted position at death characterized by positioning the abdomen under the thorax with the cephalic region drawn down."

Other Effects

Developmental Aspects. In a review by Barbosa and Peters (46), several reports (prior to 1971) are noted which included observations on adverse effects of photoactive substances on insect development. Growth-rate retardation appears to be one of the more commonly observed effects. Prolonged periods of larval development and undersized pupae have been reported. Some workers speculated in these early studies, and more recently (37-38), that phototoxins inhibit maximum utilization of energy sources or cause lethal energy stresses in the organism.

In more recent studies, decreased body weights of insects sensitized with substituted xanthene dyes have been documented. Correlations between the decrease in body weight in the boll weevil and efficiency of dyes (increasing halogenation) were reported by Callaham et al. (47) as rose bengal > phloxin B > erythrosin B > eosin Y. Further documentation of rose bengal affecting body weight in the boll weevil was made by Callaham et al. (38); untreated insects showed weight gains of 30% while weights of treated weevils remained essentially the same. Growth-inhibition in the house fly treated with rose bengal and erythrosin B has been investigated (48); larvae reared on treated medium showed inhibition of pupation and decreased pupal weights.

Clement et al. (49) examined the effect of rose bengal on development of larvae of the black cutworm (Agrotis ipsilon (Hufnagel)). Using the number of fecal pellets produced, they found that treated larvae produced significantly fewer pellets than controls. Retarded larval growth observed in this study may have been the result of temporary inhibition of ingestion.

Insect growth regulators (IGRs) are currently a new technology being developed for insect control. There is considerable evidence that some phototoxins affect insects similarly. Barbosa and Peters (46) in their review mention descriptions of developmental abnormalities of sensitized insects resembling those that more recently have been noted in IGR-treated insects. Neutral red was reported to cause 80% mortality in elaterid larvae as the moulting phase began (50). Morphological abberrations were noted in larvae of the butterfly Colias eurytheme (Bois.) when fed diets of neutral red. In a related species (C. philodice (L.)), neutral red caused pupal deformations. Bridges et al. (51) tested a new fluorescent compound against larvae of Aedes aegypti (L.) and found it produced morphogenetic effects similar to methoprene. Larvae failed to complete the moulting process and larval-pupal intermediates were formed. Specimens that formed apparently normal pupae often died before the adult emerged or died only partially emerged. Adult males that emerged normally did not complete genitalia rotation. Morphological abnormalities in larvae and pupae from rose bengal-treated mosquito larvae have been suggested to result from improper formation of chitin (52) or from a dye-induced energy stress on the insect (36). The IGR effect has been observed in pupae of the face fly when larvae were treated with rose bengal and erythrosin B (53). Most flies died in the pupal stage as the adult attempted to emerge from the puparium; some morphologically normal flies emerging from erythrosin B-treated manure had shorter life spans than the controls.

Downum et al. (54), in a study on the phototoxic effects of alpha-terthienyl on the tobacco hornworm (Manduca sexta (L.)), noted drastic developmental alterations in treated larvae. Dietary alpha-terthienyl, with irradiation of larvae after ingestion, resulted in delayed and abnormal pupal formation with no subsequent adult emergence; additionally, larval growth was delayed for four days after treatment when larvae refused diet. Pronounced tissue necrosis was observed at application sites of topically treated, irradiated larvae; at pupation, normal sclerotization and melanization were affected in various areas of the pupal case.

Reproductive Aspects. There are apparently only a few reports of the adverse effects of photodynamically active substances on the reproductive potential of insects. In an early report, David (55) speculated that methylene blue was "somehow affecting gametogenesis" of Drosophila in a study of the effects of this dye on successive generations. In a later study, David (56) observed that methylene blue caused a marked decrease by a factor of four in the fecundity of treated Drosophila females.

More recently, Pimprikar et al. (57) reported that fecundity

in the house fly was directly related to the dietary concentration of rose bengal and the frequency of dye feeding; up to 70% reduction in fecundity was observed in females maintained on a continuous diet for 15 days. As noted in the previous section, several studies have shown that pupal and adult weights of some insects are decreased by treatments with xanthene dyes. However, no observations were made in these studies on how these developmental abnormalities might affect reproduction. Since it is known for some insect species that a correlation exists between pupal weight and number of eggs produced by females, it seems reasonable to assume that sensitized insects producing abnormally smaller pupae or adults which fail to show normal weight gains may not be capable of producing a normal complement of eggs.

Ovicidal properties of phototoxins have not been studied extensively. Pimprikar et al. (57) investigated the ovicidal activity of six xanthene derivatives against the house fly. Rose bengal and erythrosin B were most active causing nearly 30% inhibition of egg hatch while rhodamine B and eosin Y were the least active causing about 15% inhibition each. Typical toxic symptoms observed by the authors were: some eggs failed to hatch (presumably due to embryo death prior to hatch); some larvae eclosed from a longitudinal line of weakness at the mesal dorsum of the chorion; some larvae freed the head capsule, but were unable to free the caudal end from the chorion.

Kagan and Chan (58) studied the photoovicidal activity of three naturally occurring molecules against D. melanogaster. Phenylheptatriyne, alpha-terthienyl, and 8-methoxypsoralan all prevented egg hatch. The first two were toxic to eggs in the dark, but upon irradiation, effectiveness was increased 37 and 4,333-fold, respectively.

Summary and Remarks

In general, we know far less about the physiological effects of photodynamic action on insects than we do about this phenomenon as it affects the physiology of other invertebrates and mammals. This is especially true at the subcellular and cellular levels. At the systems level, we have gained considerable knowledge during the last decade of how phototoxins can modify the biochemical processes and physiology of insects. But still, only a limited number of studies have been done at this level, particularly with the nervous system; at this point in time we can only speculate from abnormal behavioral patterns observed in sensitized insects that pertubations are occurring in the nervous system.

There is strong evidence from recent studies that some photoactive substances can modify the physiological processes in insects in much the same manner as IGRs. There appears to be a need for further study on how the phototoxins affect the reproductive potential of insects; many IGRs have been shown to reduce fecundity and induce sterility, but only a couple of recent studies have dealt with this aspect in any detail using the more effective phototoxins. In studies where pupal and adult weights

were observed to be altered in sensitized insects, there was no followup as to what effect these abnormalities may have on reproductive ability.

It has been suggested that the photodynamic action mechanism may not be a viable option for insect control (15). Recognizing that all the easy chemistry on insecticides has been done, isn't it time to explore phototoxins as an alternative to, or possible use in an adjunctive role, to the more conventional insecticides? Whatever some may think about investigating "insidious and little-understood mechanisms (of phototoxins) to rescue ... a borderline technology" (15), it should remain a challenge to researchers to develop that technology to combat insect pests. The more we understand about the modes of action of photodynamically active substances, the more intelligently we will be able to use them to our benefit.

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