Light-Activated Pesticides

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Light-Activated Pesticides

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Foreword

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

Preface

W ORLD AGRICULTURAL NEEDS HAVE EXPANDED as world population has
expanded. The pressures on agricultural productivity caused by pests (e.g.,
insects, weeds, and fungi) are becoming critical. At the same time,
deregistration of pesticides because of safety considerations and loss of the
efficacy of pesticides because of resistance threaten existing control
methods.

Although the catalytic action of light on the toxicity of certain
chemicals in biological systems has been known for almost a century,
exploitation of this mechanism as a watershed for new pesticides began in
earnest around 1970. Since then, a rapidly increasing interest in the
approach has led to the development of compounds active against
agricultural pests. The first patents were issued recently, and commercial
products were registered.

At the same time, scientists working somewhat independently of one
another in such diverse fields as synthetic dyes, natural products, and
chemical intermediates that lead to photodynamically active chlorophyll
derivatives were building research programs. The symposium from which
this book was developed was originally intended to be a forum in which
these scientists could meet and discuss their results, cross-fertilize ideas, and
enlighten those not comfortably conversant with light-activated pesticides.
The book grew out of the fact that no single comprehensive treatment of
light-activated pesticides existed, although portions of the topic had been
treated elsewhere. We would like this volume to serve as a single source for
anyone interested in obtaining state-of-the-art knowledge of light-activated
pesticides as well as the fundamental principles upon which the topic is
built. Comprehensive chapters should enable any interested scientist to
develop a complete library of the original literature upon which the
chapters are based. We hope that this book becomes a "bible" for anyone
interested in light-activated pesticides.

Division of Agrochemicals of the American Chemical Society for sponsoring the forum. We also thank the authors for providing quality chapters in a professional and timely manner. Finally, the quality of any book depends to some extent on the quality of anonymous reviews. We thank the reviewers whose invaluable suggestions strengthened the individual chapters.

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Chapter 1

Development of Photoactivated Compounds as Pesticides

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Although light has been known to enhance certain
toxic reactions since 1888, the principle was not
exploited until after 1970 to any great extent.
The greatest concentration of effort has been in
the study of photodynamically active dyes, pri-
marily the halogenated fluorescein series, as
prospective insecticides. More recently, compounds
of plant origin have been isolated, identified, and
studied as phototoxins against a wide range of pests,
including insects, fungi, and weeds. The main classes
studied to this time are the furanocoumarins, thiophe-
nes, acetylenes, extended quinones, and the
chlorophyll a intermediates popularized as "laser
herbicides." It is apparent that this area of
research will expand in the coming years rather than
retrench.

The expenditure of energy frequently helps to enhance the prob-
ability of successfully reaching one's goals in this universe. For
as long as chemistry has existed as a science, we have input
energy, most frequently heat energy, into chemical reactions to
make the molecules or to produce the effects which we wanted. The
use of light energy has remained quantitatively a minor component
as a means of energy input. This has also been the case with the
development of the pesticide industry. Light energy has not been
used to drive toxicological reactions or to provide specificity for
those reactions to any great extent until the decade of the 70's.
Several review chapters have been written covering individual
aspects of photodynamically active pesticides (1-8). The purpose
of this chapter is to provide a chronological treatment of the
development of light as an integral part of the toxicological
action of several classes of pesticides; and also, to show the
development of the various classes of light activated pesticides
relative to each other.

Early History

The first documented study in which light was understood to cause
an enhancement of a chemically induced toxic effect was that of
Marcacci (9) in which he reported that alkaloids were more effec-
tive against seeds, plants, fermentations, and amphibian eggs in
sunlight than in the dark. Rabb (10) subsequently reported that
sunlight caused an increase of several orders of magnitude in the
acridine sensitized mortality of paramecia. Paramecia exposed to
acridine in the dark and paramecia exposed to the sun in clear
water were not nearly as vulnerable. By 1904, Jodlbauer and von
Tappeiner (11) had demonstrated the requirement for oxygen and had
coined the term "photodynamic action." Much later, Spikes and Glad
(12) would operationally define photodynamic action as the killing
or damaging of an organism, cell, or virus or the chemical modifi-
cation of a molecule in the presence of a sensitizing dye and mole-
cular oxygen. One problem in the early development of light
activation of molecules as a contributor to a deleterious effect
or even the death of a living specimen was that light was not con-
sidered as an experimental parameter. Therefore, it is difficult
to scan the early literature for examples simply because the light
intensity was usually uncontrolled and unreported (13-27).
     The first reported use of photodynamic action against an
insect target was that of Barbieri (28) in which Anopheles and
Culex mosquito larvae were shown to be susceptible to solutions of
several classes of dyes in direct sunlight. The most active dyes
were the halogenated fluorescein derivatives, erythrosin and rose
bengal, alone and in mixture (I). There were no deaths reported from
either dye-treated, non-light-exposed populations or non-dye-
treated, light-exposed populations.
     The approach lay dormant until 1950, when Schildmacher (29)
treated Anopheles and Aedes mosquito larvae with a series of dye
solutions and exposed them to sunlight. Conducting field tests
in small ponds and at least on bomb crater left over from World
War II as well as in laboratory tests, he reported that rose
bengal was more toxic than erythrosin and that eosin and
fluorescein were ineffective. Schildmacher also made the first
attempt at the definition of the toxicological target when he
reported that the midgut epithelial cells showed considerable
damage after light exposure. Finally, he observed that photodyna-
mic action had no effect on the mosquito fish (Gambusia sp.) that
were present.
     In order to put these findings into perspective, one should be
aware of the state of the art in pesticide technology at this time.
Ware (30) listed the following as some of the important milestones
during this period. Pyrethrum was introduced into Kenya (1928).
Methyl bromide (1932), pentachlorophenol (1936), TEPP (1938),
Bacillus thuringiensis (1938), DDT (1939), hexachlorocyclohexane
(1941), 2,4-D (1942), warfarin (1944), chlordane (1945), toxaphene
(1947), malathion (1950), and Maneb (1950) were either discovered
or introduced. At this time, the primary criterion for a pesticide
was its toxicity, as Rachel Carson would not write "Silent Spring"
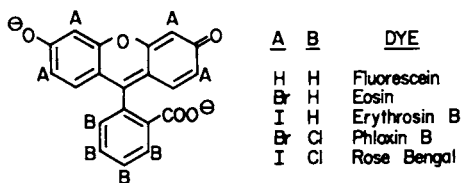for another decade.

     During this same time also, biochemists and photobiologists
became interested in the mechanism of dye sensitized photooxidation
and its effect on living cells and cellular components. Many
excellent reviews have been written on the subject (31-37).
Photosensitization has been shown to occur by one of two mecha-
nisms: Type I and Type II. The initial step in the photosensiti-
zation process is the absorption of visible or UV light by the
sensitizer. In the Type I mechanism, the excited sensitizer con-
verts the substrate to product via free radical intermediates
including oxygen. In the Type II mechanism, the excited sensitizer
reacts by causing the formation of singlet oxygen which then reacts
with the substrate, thereby converting it to the oxidized product.

Dye Insecticides

The concept lay dormant again until 1971, when a group at West
Virginia University, Yoho, Butler, and Weaver, then published the
first of several investigations of the efficacy of photodynamic
action against the adult house fly using primarily the halogenated
fluorescein series of dyes (38). These papers, based substantially
on the dissertation of Yoho (39), compared toxicological data with
the parameters of light source and intensity, dye structure and
conventration in the diet, source of light, and length of light
exposure (38,40). Later, Yoho et al. (41) studied a series of 14
Food, Drug and Cosmetic dyes for efficacy in photodynamic toxicity
to house fly adults. It was also reported in the dissertation that
the midgut epithelial cells appeared to be damaged and that the
external symptoms associated with toxicity suggested an involvement
with the nervous system. It can fairly be said that the great
majority of the work on photosensitizing dyes as insecticides can
be traced back to the first paper in this series as the watershed.
After its publication, there came a deluge of interest in this
area.
     Graham et al. (42) reported that with the methylene blue sen-
sitized phototoxicity of yellow mealworms, the intensity of
sunlight was much more than required to obtain adequate effec-
tiveness. Yoho et al. (40) attributed the lower toxicity of methy-
lene blue (II) in fluorescent light relative to sunlight to the poor
overlap with the absorption spectrum in the former case.
     Broome et al. (43,44) reported on the toxicity of a series of
xanthene dyes with the black imported fire ant where mortality was
compared with dye structure, incubation period in contact with the
dye, dye concentration in the feed and in the insect, continuity of
light exposure, light intensity, and exposure time. Although there
was no mortality observed in the imported fire ant after 3 days of
exposure to rose bengal in the dark, they did observe an onset of
mortality that eventually resulted in an LT_{50} value of 8.4 days.
This may be compared with an LT_{50} value of 0.7 hr for adult fire
ants exposed to $3800 \mu W/cm^2$ from a Cool White fluorescent light
after 24 hr exposure to the rose bengal in the dark (Broome et al.
(44). This observation led to the acceptance of the dark reaction
as a second, though admittedly much less efficient, toxic mechanism
caused by certain photodynamic dyes in insects. Quantitative study
of the dark reaction with adult life stages of the boll weevil

A	B	DYE
H	H	Fluorescein
Br	H	Eosin
I	H	Erythrosin B
Br	Cl	Phloxin B
I	Cl	Rose Bengal

Structure I

Methylene Blue
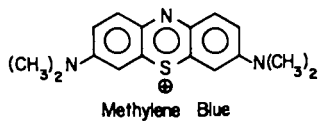
Structure II

(45), the face fly (46), and the house fly (47) showed the
widespread occurrance of this toxic mechanism. In fact, David and
Heitz (48) reported on an imported fire ant field control scheme
based on a phloxin B-impregnated bait where the control reported
was almost certainly due to the dark mechanism working deep within
the nest.
    At about this same time, mechanism studies were appearing. The
acetylcholinesterase from the black imported fire ant (49) and the
boll weevil (50) was susceptible to dye-sensitized photooxidation
in vitro but levels were not depressed in insects killed by photo-
dynamic action. Weaver et al. (51) reported that in the cockroach,
photodynamic action caused a significant decrease in the hemolymph
volume and a large increase in the crop volume. Later, Weaver et
al. (52) showed that erythrosin B-sensitized photodynamic action
caused a reduction of hemocytes relative to controls. At the
highest injected levels in the dark, there was also observed a
reduction in hemocytes, which is probably due to the dark toxic
mechanism. In the absence of light, Broome et al. (53) reported
that in boll weevils fed rose bengal during larval development,
there were decreases in the wet weight, dry weight, protein levels,
and lipid levels of the adult insect. Later, Callaham et al. (54)
showed that the lower levels were due to a lack of growth after
adult emergence in the treated insects. This was interpreted as an
energy drain caused by the presence of the dye in the adult tissue.
    In 1978, Fondren et al. (47) compared the toxicities of 6
xanthene dyes to the house fly in terms of both dietary and tissue
levels of the dyes in question. Indications of feeding inhibition
were observed at high dye concentrations in the food. Although
species differences were observed when the house fly data was com-
pared with similar boll weevil data, it was reported that, in
general, the effectiveness of the dyes was most dependent on the
phosphorescence quantum yield than any other physico-chemical para-
meter. Similar interpretations were made in a later study of the
face fly (46).
    In a study of light intensity as a critical parameter in the
photodynamic toxicity of rose bengal to the adult house fly,
Fondren and Heitz (55) showed that the accumulated number of pho-
tons needed to kill 50% of a population decreased as the intensity
increased. This would infer that there is a regenerative capacity
within the insect that is more efficiently overcome by photodynamic
action as the light intensity increases. Light source was also
studied as an experimental parameter (56) where it was shown that
sunlight was more efficient than fluorescent light due to the
larger number of photons striking the target; but it was also
shown that fluorescent light was more efficient than sunlight due
to the better overlap of the lamp output with the absorption
spectrum of the xanthene dyes. Lavialle and Dumortier (57)
reported that methylene blue-fed larvae of the cabbage butterfly
were susceptible to photodynamic action after exposure to either
fluorescent light or sunlight. Mortality was shown to be dependent
on dye concentration, light intensity, duration, and wavelength.
In laboratory toxicity tests using several xanthene dyes against
the black cutworm, Clement et al. (58) found that rose bengal was

the most effective and that toxicity was directly dependent on the
light intensity. In this case, the larvae avoids the light and
that makes this particular application undesireable. Creighton
et al. (59) reported on the toxicity of rose bengal to the cabbage
looper, the corn earworm, and the pickleworm. Photodynamic action
was relatively ineffective under these conditions, but the dark
toxicity was observed.

In 1979, Pimprikar et al. (60) began reporting on an extended
series of tests with mosquito larvae. Under fluorescent light and
at rose bengal treatment levels of 1 to 20 ppm, Culex larvae were
more susceptible than Aedes larvae and early instars were more
susceptible than later instars. Physiological and morphological
abnormalities were observed in the pupal and adult stage after
larval stage treatment which suggested improper chitin formation in
the insect. This sometimes resulted in incomplete extrication of
the pupal stage from the larval cuticle and of the adult stage from
the pupal cuticle. Where this was observed, mortality resulted.
They also reported the observance of larval-pupal intermediates
similar to those observed after treatment with insect growth regu-
lators.

Pimprikar et al. (61) attempted to control house flies in a
commercial caged layer house using weekly applications of aqueous
solutions of erythrosin B directly on the manure. In a duplicated
5 week treatment period, they reported decreases of adult and lar-
val house flies up to 90% with respect to pretreatment levels with
no change in the beneficial soldier fly larval population. The dye
was reported to be somewhat rapidly degraded in the manure illu-
minated by indirect sunlight such that only about 20% was extrac-
table 1 week after spraying. As a result of these tests, Pimprikar
et al. (62) studied the effects of several fluorescein derivatives
on each developmental stage of the house fly. Treated adults exhi-
bited lowered fecundity, the eggs exhibited a reduced viability.
and mortality was observed in each life stage of the house fly.

Carpenter and Heitz (63) showed that when larval mosquitoes
were exposed to rose bengal and visible light, significant acute
mortality was observed. Further, if the treated mosquitoes were
illuminated with visible light and then put into darkness, a latent
mortality was observed. The light treatment was necessary to
obtain the latent mortality, as the controls exposed to the same
dye concentrations in the dark exhibited no latent mortality. When
the latent mortality was added to the acute mortality, it was
observed that the total toxicity of the rose bengal was increased
by 10-fold over the dark toxicity. Later, Carpenter and Heitz
(64) studied the relationships between the slow, light-independent
mechanism, the rapid, light-dependent mechanism, and the slow,
light-initiated, latent mechanism during the treatment of Culex
larvae with erythrosin B. Quantitative analysis was hampered by
the photodegradation of the erythrosin B during the time course of
the study which made the expression of toxicity relative to dye
concentration impossible.

Fairbrother et al. (65) made a very complete study of the toxi-
cological effects of erythrosin B and rose bengal on the face fly.
When larvae developed on manure into which either dye was incor-
porated, either by hand or by passage of the dye through cattle,
mortality was observed at each life stage. Some of the flies died
at various stages of emergence as if the effort associated with
emergence was too stressful. Several of the adults were unable to
complete the extrication from the puparium and were stuck to the
chitin inner lining of the puparium. Others had deformed wings.
Adults, held from emergence and illuminated with visible light,
were observed to have a much higher mortality than controls, thus
suggesting that dye sequestered in the insect body during develop-
ment from larvae to adult was responsible for the toxicity. This
is the first report of photodynamic action occurring in a life
stage different from the life stage which ingested the dye.

Carpenter et al. (66) reported that the presence of fluorescein
enhanced the toxicity of rose bengal in photodynamic reactions in
Aedes larvae. This synergism was initially explained by the utili-
zation of photons absorbed by the fluorescein molecule that were
not of the proper wavelength for absorption by the rose bengal
molecule. A United States patent was issued covering the synergism
of a nontoxic dye with a demonstrated toxic dye in both house fly
and mosquito systems (67). Later, in tests involving 8 xanthene
dyes, it was not possbile to confirm the mechanism of action as
that referred to above (68). Further, the synergism could not be
correlated with the number of halogens, percent halogenation, mole-
cular weight, partition coefficient, fluorescence quantum yield of
the synergist dye, or the overlap interval for the synergist dye
with erythrosin B. The mechanism of action of the synergism
observed with the xanthene dyes is still unexplained.

Sakurai and Heitz (69) studied the inhibition of growth and the
photodynamic action caused by rose bengal and erythrosin B in the
house fly. Larvae reared in the dark on agar containing either dye
exhibited a concentration dependent decrease in pupation rate and
in adult emergence. House flies which had consumed a nonlethal
amount of dye in the larval stage exhibited a considerable light-
dependent toxicity in the adult stage. It was also observed that
pupae injected with rose bengal developed into adults which were
more susceptible to photodynamic action than adults injected with
the same dye. Further, the susceptibility of the injected adults
was comparable to adults fed the dye, thus suggesting that the ali-
mentary canal may not be the only site of action as suggested pre-
viously (29,39,51).

In 1983, Respicio and Heitz (70) began a study of the develop-
ment of resistance to erythrosin B in the house fly. A laboratory
strain developed only 6-fold resistance after 40 generations of
challenge by erythrosin B. This low level of resistance was due to
the inbred quality of the laboratory strain. A new, wild strain
developed a 48-fold resistance after 32 generations of exposure to
increasing levels of erythrosin B in the diet. Upon removal of the
selection pressure for 20 generations, the resistance remained
constant. Reciprocal crosses showed that the resistance is
inherited as a codominant character and that sex linkage is not

involved. Later, the cross-resistance of erythrosin B-resistant
house flies was studied against strains resistant to propoxur, DDT,
permethrin, and dichlorvos (71). No cross-resistance for a dif-
ferent pesticide was observed for any of the 5 strains, with one
exception. The erythrosin B-resistant strain was cross-resistant
to phloxin B and rose bengal, but this is to be expected since they
function by the same mechanism. Recently, cross-resistance has
been shown when the erythrosin B-resistant strain was challenged by
alpha-terthienyl mediated photodynamic action (Pimprikar, G.D. and
Heitz, J.R., unpublished results).

The relative toxicities of 6 xanthene dyes to Culex and Aedes
mosquito larvae was reported by Pimprikar et al. (72). Rose bengal
was the most toxic followed by phloxin B and erythrosin B. At the
same time, it was shown that these same dyes exhibited a low toxi-
city to the mosquito fish, thereby confirming the observation of
Schildmacher (29), and did not affect the predatory efficiency of
the fish. The lack of an effect on the predatory mosquito fish
would allow the dyes to be considered in an integrated pest manage-
ment scheme.

In 1984, Pimprikar and Heitz (73) observed an unusually high
insecticidal activity in Aedes mosquito larvae which had been illu-
minated after exposure to the insoluble free acid forms of the
xanthene dyes. In all previous studies, the larvae had been
treated with the water soluble salt forms of the dyes and the
larvae consumed the dye as they ingested the water. With the
insoluble dyes, they were able to filter feed on dye particles and
thereby receive a higher level of dye. Toxicity ratios ranged up
to 2 orders of magnitude between the soluble and insoluble forms of
the same dye. In a later study, Carpenter et al. (74) showed that
the insoluble forms of the xanthene dyes were 10-fold more
effective against Culex mosquito larvae than the soluble forms.
Further, they reported that when the insoluble forms of the dyes
were dispersed with a surfactant, such as sodium lauryl sulfate,
the dyes were 50- to 60-fold more effective than the soluble forms.
Respicio et al. (75) studied the toxicity to Culex mosquito larvae
of coprecipitated free acid, nondispersible and dispersible for-
mulations of fluorescein and erythrosin B. The 1:1 combination of
fluorescein:erythrosin B, dispersed with sodium dodecyl sulfate,
was the most toxic formulation and also showed synergistic action.
In a more detailed study of the synergistic effect, they showed
that the 1:1 mixture of fluorescein:rose bengal was more toxic than
the 3:1 mixture, but the 3:1 mixture exhibited more synergism
(Respicio, N.C., Carpenter, T.L., and Heitz, J.R. J. Miss. Acad.
Sci, in press). Carpenter et al. (76) evaluated a series of 8
dispersants for use with the insoluble forms of the dyes and none
were toxic alone. Erythrosin B, dispersed with sodium dodecyl
sulfate, was the most toxic against Culex mosquito larvae. In
small-scale field tests, this formulation caused significant
reductions in larval and emergent adult populations of Culex
mosquitoes at concentrations ranging from 0.25 to 8.0 ppm.

Not all of the work with the fluorescein dyes concerned
insects. In 1985, Knox and Dodge (77) reported on the photodynamic

action of eosin on pea leaf tissue. Chloroplasts were shown to be
particularly sensitive to visible light after eosin treatment. The
treated tissue exhibited lowered photosynthetic oxygen evolution,
lowered photosynthetic electron transport capability, lowered
levels of ribulose-bisphosphate carboxylase and NADPH-dependent
glyceraldehyde-3-phosphate dehydrogenase, and pigment loss. The
initial loss of photosynthetic activity was associated with damage
to the thylakoid membranes. In an accompanying paper, Knox and
Dodge (78) further characterized the site of the photodynamic
action in pea leaf tissue as photosystem II.

Robins and Beatson (79) attempted to protect house fly larvae
against erythrosin B sensitized phototoxicity. Beta-carotene pro-
tected, but butylated hydroxytoluene, ascorbate, and diazabi-
cyclooctane actually enhanced the toxic effect.

Hawkins et al. (80) showed that erythrosin B and visible light
(from either fluorescent sources or sunlight) were toxic to the
infectious 3rd stage larvae of gastrointestinal nematodes of
naturally infected cattle. Toxicity was significant after only 2
consecutive daily oral treatments of the cattle. Later, they
reported that the photodynamic action was ineffective against the
adult stage viability or fecundity (Hawkins, J.A.;
Johnson-Delivorias, M.H.; Heitz, J.R. Veterin. Parasitol., in
press). There was a consistent effect on the 3rd stage larvae
which was dependent upon dosage, time of light exposure and, to
a lesser extent, the length of time the larvae were left in the
presence of the dye.

Photoactive Plant Components

In the study of the photoactive dyes, the research was focused pri-
marily on a deep understanding of the mechanisms of action of only
a small number of dyes from predominately one class of compounds.
In the study of photoactive plant components, there has been a
shift of emphasis. Much of the research has been aimed at isola-
tion and identification of novel plant components, delineation of
the general mechanisms of action and the type of sensitive organism.
As such, there are fewer papers on any given compound, but many
more compounds studied. Some of the major classes of plant derived
compounds will be examined here. Recently, an entire issue of the
Journal of Chemical Ecology was devoted to the invited papers pre-
sented at a symposium on interactions between insects and photo-
active plants presented at the 1984 national meeting of the
Entomological Society of America (81). Several related papers were
also included which were not part of the symposium.

Furanocoumarins

Furanocoumarins have been implicated in certain phototoxic respon-
ses in grazing cattle (82). In 1978, Berenbaum (83) reported that
when the linear furanocoumarin, xanthotoxin (III), was administered to
the larvae of the southern armyworm, a low level of toxicity was
observed that was greatly enhanced when UV light was shown upon the
larvae. She also observed a longer time required for pupation to

occur in those larvae that did not die. The biological activity of
the furanocoumarins are due to the intercalcation of the molecule
into the double stranded DNA where, upon activation by UV light,
covalent bonds are formed with pyrimidine bases (84). Song and
Tapley (85) demonstrated that the mechanism of action was Type I in
which oxygen radicals are involved. Later, Berenbaum and Feeny
(86) reported that the angular furanocoumarin, angelicin (IV), reduced
the growth rate and the fecundity of the larvae of the black
swallowtail butterfly, whereas xanthotoxin was not appreciably
toxic to this insect. It was interpreted that the angular forms of
the furanocoumarin were later evolutionary developments which
helped to protect the plant from insect herbivory.
      Recently, Ivie et al. (87) in an initial report on the
metabolism of furanocoumarins by black swallowtail butterfly
larvae, showed that this insect detoxifies this class of com-
pounds by metabolism in the midgut tissue prior to absorption. In
this manner, appreciable levels of unmetabolized furanocoumarin do
not enter the body circulation. Later, it was reported that the
increased phototoxicity of the angular furanocoumarins relative to
the linear furanocoumarins was due to a slower rate of hydrolysis
of the furan ring of the angular derivatives (88,89).
Ashwood-Smith et al. (90) reported that the black swallowtail
larvae were able to degrade xanthotoxin into biologically inactive
compounds. The enzyme reaction required an electron generating and
accepting system similar to the mixed function oxidases of mam-
malian microsomes. In vitro studies of the relative metabolic
rates of hydrolysis of xanthotoxin by homogenates of last stage
larvae of the black swallowtail butterfly and the fall armyworm
showed that the former insect hydrolyzed the xanthotoxin 6 times
faster than the latter insect (91).

Alpha-Terthienyl and Polyacetylenes

Alpha-terthienyl (V) was shown to be nematicidal by Uhlenbroek and
Bijloo (92). Gommers (93) reported that irradiation with near UV
light strongly enhanced the nematicidal activity of alpha-
terthienyl. Later, Gommers and Geerligs (94) showed that endopara-
sitic plant nematodes which had been exposed to alpha-terthienyl in
the roots of African marigolds for 10 days were rapidly killed upon
exposure to near UV light. Bakker et al. (95) demonstrated that,
upon irradiation, alpha-terthienyl generates a reactive oxygen spe-
cies, probably singlet oxygen, upon which the nematicidal activity
depends. Gommers et al. (96) reported that the irradiation of
alpha-terthienyl required aerobic conditions for the rapid killing
of nematodes. In vitro studies of enzyme inhibition and protection
by a series of singlet oxygen quenchers further supported the
hypothesis that the active oxygen formed in the reaction was
singlet oxygen.
      Alpha-terthienyl and phenylheptatriyne (VI) were shown to be power-
ful toxic photosensitizers against first and fourth instar Aedes
mosquito and blackfly larvae in both sunlight and UV light (97,98).
The mode of action of alpha-terthienyl was shown to be photodynamic
in nature but that of phenylheptatriyne-type compounds was not as
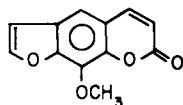
Xanthotoxin

Structure III
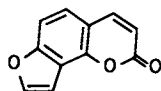
Angelicin

Structure IV
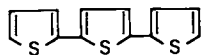
α – Terthienyl

Structure V

l-Phenyl-1, 3, 5-heptatriyne

Structure VI

clear (99). McLachlan et al. (100) investigated structure-activity
relationships for a series of polyacetylene and thiophene deriva-
tives against a bacterium and a yeast with the thiophenes being
generally more toxic than the acetylenes. Activity was directly
dependent upon the number of thiophene rings and acetylene bonds.
There was a positive correlation between phototoxicity and the
octanol-water partition coefficient; but there was little
correlation with photon absorption.

The dose response in relation to the light source was studied
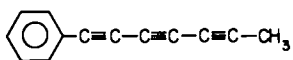by Arnason et al. (101). Although alpha-terthienyl exhibited low
toxicity in the absence of light, the enhanced toxicity to Aedes
mosquito larvae upon irradiation by near UV light led to its
investigation as a commercial larvicide in field trials using simu-
lated small ponds. Alpha-terthienyl was even more toxic to the
mosquito larvae in sunlight. An action spectrum showed that there
was good agreement between light absorption and toxicological
action.

In 1983, Kagan and Chan (102) showed that both phenylhep-
tatriyne and alpha-terthienyl displayed ovicidal activity against
the eggs of the fruit fly in the dark. They reported that irra-
diation by long wavelength UV light enhanced the toxicity by 37-
and 4333-fold, respectively. Using the singlet oxygen dependent
conversion of adamantylidene adamantane to adamantanone, Kagan et
al. (103) were able to compare the relative singlet oxygen
generating capability of a series of thiophene derivatives.

The polyacetylenic compound, cis-dehydromatricaria ester was
shown to be ovicidal to freshly laid eggs of the fruit fly. Upon
irradiation with ultraviolet light the ovicidal activity was
enhanced (104).

Later, Downum et al. (105) reported that the tobacco hornworm,
when given a single ingested dose of alpha-terthienyl followed by
exposure to UV light, exhibited delayed and abnormal pupal for-
mation with no subsequent adult emergence. Topical application of
alpha-terthienyl followed by irradiation with near UV light
affected both the sclerotization and melanization of the pupal case
in later development.

Kagan et al. (106) demonstrated the first example of the inac-
tivation of acetylcholinesterase in vivo by a photoactive pesticide
when they showed that alpha-terthienyl, as well as 3 isomers,
caused the inhibition of this enzyme in Aedes mosquito larvae upon
UV light irradiation. Later, Reyftmann et al. (107) showed that
alpha-terthienyl exhibited a very long-lived excited triplet state
which allowed it to react very favorably with oxygen, thereby pro-
ducing singlet oxygen. Since it does not react well with hydrogen
or electron donors, it appears that alpha-terthienyl functions pri-
marily as a Type II photodynamic agent.

Kagan et al. (108) studied the phototoxic effects of alpha-
terthienyl on fathead minnows and it was found to be at least twice
as potent as rotenone and nearly as potent as endrin.

In 1985, a Canadian patent was awarded to Towers et al. (109)
covering the control of pests (algae, fungi, nematodes, or her-
bivorous invertebrates) by polyacetylenes. Activation of the
polyacetylene by the UV component of sunlight enhanced the toxic
effects observed in the absence of light.

Hypericin and Cercosporin

It has been known for many years, that when grazing animals feed
on certain members of the plant genus Hypericum, they
become sensitive to sunlight. This sensitivity is accompanied by
intense skin irritation and inflammation which may become fatal.
Horsley (110) showed that this condition was caused by hypericin (VII),
a highly condensed quinone (111,112). Yamazaki et al. (113) noted
the similarities between the structures of hypericin (VII) and cerco-
sporin (VIII). When they exposed cercosporin-treated mice and
bacteria to light, mortality was observed. Cercosporin also was
shown to damage plant tissue under illumination by incandescent
light (114). Daub (115) reported that the kinetics of the killing
of tobacco plant cells was a function of cercosporin concentration,
light intensity, light wavelength, and singlet oxygen quenchers.
Since the toxic response was inhibited by Dabco and bixin, known
quenchers of singlet oxygen, it was proposed that cercosporin
produced singlet oxygen and functioned as a photodynamic
agent. Later, Daub (116) showed that cercosporin-caused
electrolyte leakage from tobacco leaf discs was probably due to
lipid hydroperoxide formation from membrane lipids. Cercosporin
was shown to oxidize solutions of methyl linolenate, while alpha-
tocopherol had an inhibitory effect on the cercosporin-mediated
lipid peroxidation. Daub and Briggs (117) then showed that
the unsaturated acyl chains of lipids were the target of the
photodynamic action. When the unsaturated acyl chains are oxidized,
spin labelling experiments showed that the membranes become more
rigid at all temperatures and that the membrane phase transfor-
mation temperature increased from 12.7° to 20.8°C. In 1983, Daub
and Hangarter (118), reported that cercosporin produced superoxide
radicals as well as singlet oxygen upon exposure to light in the
presence of oxygen. Cercosporin reacted with cholesterol to form
the 5 alpha-hydroperoxide of cholesterol. This reaction is speci-
fic for singlet oxygen. Cercosporin also reduced p-nitro blue
tetrazolium chloride which is readily reduced by superoxide.
Superoxide dismutase, an enzyme which reacts very rapidly with
superoxide, inhibited this reaction.
    In 1985, Knox and Dodge (119) isolated hypericin from the hairy
St. John's wort and showed that it sensitized the photooxidation of
methyl linolenate. The reaction was inhibited by the carotenoid,
crocin. Hypericin was shown to produce singlet oxygen due to oxy-
gen consumption during the sensitized photooxidation of imidazole
and also due to inhibited rates of oxygen consumption during the
reaction in the presence of deuterium oxide or sodium azide.
Hypericin also caused pigment loss and ethane production from pea
leaf discs under light exposure.

Laser Herbicides

Since many pesticides are discovered as a result of extensive
screening programs of many candidate chemicals, it is really not
necessary to understand the mechanism of action of the candidate

pesticide at first. Rather, it is necessary only that it be effec-
tive. There does exist in the realm of the family of photoacti-
vated pesticides, a herbicide which was actually designed on the
basis of knowledge of the inherent biochemical pathways in plants.

In 1969, Ellsworth and Aronoff (120) initially proposed that
chlorophyll was biosynthesized via 2 alternate parallel pathways
involving monovinyl derivatives and divinyl derivatives. Over the
next several years, Rebeiz and his coworkers studied chloroplast
biogenesis in plants (4,121). They later proposed that each alter-
nate parallel pathway contained parallel subpathways utilizing
fully esterified derivatives and acidic derivatives. They realized
at the time that the known mode of action of no herbicide took
advantage of this aspect of plant biosynthesis. If chlorophyll
biosynthesis was used as the target for the herbicidal action, it
would allow for a certain specificity. Further, the diversity of
chlorophyll a biosynthetic pathways allowed for diversity in
design. The mechanism of action of the herbicide would be based on
the photodynamic activity of the porphyrin (tetrapyrrole) derivati-
ves which are part of the chlorophyll a biosynthetic scheme.
Therefore, it would be dependent on the biosynthesis and accumula-
tion of the tetrapyrroles by the sprayed plant targets. Further, a
post-spray period of darkness of several hours would be required for
the accumulation of the tetrapyrroles. Finally, upon exposure to
light, a very damaging photodynamic effect, catalyzed by the accu-
mulated tetrapyrroles, would occur which will result in the death of
the plant target.

In order to stimulate the biosynthesis of tetrapyrroles in the
plant target, delta-aminolevulinic acid and 2,2'-dipyridyl were
sprayed on cucumber seedlings in the dark. After 17 hours in the
dark, the plants were exposed to daylight and they suffered exten-
sive photodynamic damage. The green leafy tissue and the hypocotyl
became bleached. In both cases, the tissues suffered a severe loss
of turgidity, probably due to the development of leaky cell mem-
membranes, followed by a rapid and severe dehydration of the
tissues.   Prior to light exposure, some of the seedlings were ana-
lyzed and increased cellular levels of total tetrapyrroles were
found to be concentration dependent upon the sprayed delta-
aminolevulinic acid and 2,2'-dipyridyl.

When other plants were treated similarly, it became apparent
that the delta-aminolevulinic acid and 2,2'-dipyridyl induced pho-
todynamic action causing 3 different types of herbicidal responses
depending upon the target species.

The Type I response, observed in dicots such as the cucumber,
is characterized by accumulations of tetrapyrroles in leafy
tissues, stems, and growing points, and rapid death from photodyna-
mic action which is directly dependent upon light intensity. The
Type II response is observed in other dicots such as cotton, kid-
ney bean, and soybean. Tetrapyrroles are accumulated in the leafy
tissues, but not in the stems. Leaves that accumulate the tetra-
pyrroles die very rapidly within a few hours of light exposure, but
the cotyledons, stems, and growing points remain unaffected. These
plants could recover from this initial damage by producing new
leaves. It was also observed that, if the plants were young enough

that the leaves were enclosed by the cotyledons, the plants were
completely unaffected. The Type III response was exhibited only by
monocotyledons, such as wheat, corn, oats, or barley. In this
case, the plant developed small necrotic regions when the sprayed
plant was exposed to light. The seedlings grew vigorously and
developed into healthy plants.

Rebeiz and his coworkers have thus developed the first photody-
namic herbicide. The popular press has already given this class of
herbicide another name, "laser herbicides." By whatever name they
will be called, these herbicides appear to have a promising future.
The different pathways of chlorophyll biosynthesis should allow a
degree of flexibility so that products developed from this class of
herbicides will be tolerant to the crop plants and toxic to the weeds.

In 1986, Rebeiz and Hopen were awarded a patent covering the
laser herbicide concept (122).

Miscellaneous Materials

There have been reports of other materials which may become impor-
tant as this research area develops. At this time, however, they
have not attracted the attention of the pesticide classes that were
discussed earlier in this chapter.

Maltotsy and Fabian (123-124) first found that polyaromatic
hydrocarbons were toxic to larvae of the fruitfly upon irradiation
with UV light. The high carcinogenic potential of this class of
compounds has kept them from being exploited as much as would be
expected if there were no carcinogenic risk. Kagan and Kagan (125)
addressed this problem with a comparative study the effects of
benzo[a]pyrene (carcinogenic) and pyrene (noncarcinogenic) upon
immature forms of Aedes mosquitoes held in the dark or irradiated
with UV light. Their results indicated that carcinogenicity and
phototoxicity were not inextricably linked. Later, Kagan et al.
(126) called attention to the possible deleterious effects on
aquatic organisms of polyaromatic hydrocarbons inadvertantly intro-
duced into the environment.

Kagan et al. (127) reported that 2,5-diphenyloxazole, known to
workers in scintillation counting as POP, is phototoxic to the
first instar of Aedes mosquito larvae, to crustaceans, and to the
eggs of fruitflies.   A similar compound, 1,4-bis(5-phenyloxazole-
2-yl)benzene, known as POPOP, is also toxic, but to a lesser
degree. Both can sensitize the formation of singlet oxygen.

Molero et al. (128) reported a photodynamic activity in root
tissue mediated by berberine sulfate and violet (420 nm) light. At
low concentrations (nanomolar), root growth inhibition was
complete.

The first phototoxic lignan, nordihydroguaiaretic acid (IX), from
the leaf resin of the creosote bush has been reported (Downum,
K.R.; Dole, J.; Rodriguez, E. Phytochem, in press). Many more
lignans are known, occurring in many families of plants, and they
may become an important future source of photochemically active
chemicals.

Hypericin

Structure VII

Cercosporin

Structure VIII

Nordihydroguaiaretic acid
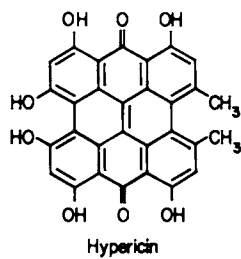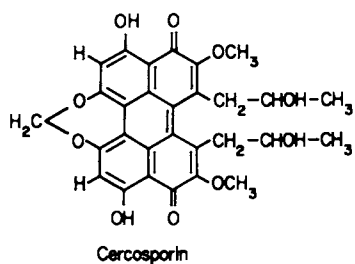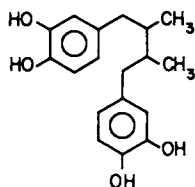
Structure IX

Conclusions

It is apparent that the concept of light activation of molecules to
enhance biological activity is a concept which is both intriguing
and currently available. Although there are applications which
would not allow catalysis by light, such as the photonegative
insects and most root tissue in plants, there is a wide and diverse
population of pests which do function in the light. The first ten-
tative steps torwards application have been taken using available
synthetic chemicals and known plant materials, all of which were
identified through general screening programs.
    It is to be hoped that the next steps may follow at least in
part the approaches of Constantin Rebeiz and his coworkers in
which the toxic molecule was designed from known priciples of the
biochemistry of the plant target. In fact, although there are no
known examples thus far, it would appear that the general area of
photoaffinity labelling of enzymes may hold promise as a watershed
for the development of new pesticides as specific targets
within the pest are more completely understood. Other fundamental
areas of light activation systems may similarly be future
watersheds for pesticides based on this approach.

Acknowledgments

Literature Cited

1. Heitz, J.R. In Insecticide Mode of Action; Coats, J.R., Ed.;
   Academic: New York, 1982; pp. 429–457.
2. Robinson, J.R. Res. Rev. 1983, 88, 69–100.
3. Arnason, T.; Towers, G.H.N.; Philogene, B.J.R.; Lambert, J.D.H.
   In Plant Resistance to Insects; Hedin, P.A., Ed.; ACS Symposium
   Series No. 208; American Chemical Society, Washington, DC, 1983;
   pp. 139–151.
4. Rebeiz, C.A.; Montazer-Zouhoor, A.; Hopen, H.J.; Wu, S.M. Enzyme
   Microb. Technol. 1984, 6, 390–401.
5. Towers, G.H.N. Can. J. Bot. 1984, 62, 2900–2911.
6. Cooper, G.K.; Nitsche, C.I. Bioorg. Chem. 1985, 13, 362–374.
7. Knox, J.P.; Dodge, A.D. Phytochem. 1985, 24, 889–896.
8. Downum, K.R. In Natural Resistance of Plants to Pests:Roles of
   Allelochemicals; Green, M.B.; Hedin, P.A., Eds.; ACS Symposium
   Series No. 296; American Chemical Society, Washington, DC, 1986;
   pp. 197–205.
9. Marcacci, A. Arch. Ital. Biol. 1888, 9, 2.
10. Rabb, O. Z. fur Biol. 1900, 39, 524–546.
11. Jodlbauer, A.; von Tappeiner, H. Muench. Med. Wochenschr. 1904,
    26, 1139–1141.
12. Spikes, J.D.; Glad, B.W. Photochem. Photobiol. 1964, 3, 471–487.
13. Edwards, W.F. Text. World. 1921, 60, 1111–1113.
14. Chang, H.T. Mosquito News. 1946, 6, 122–125.

15. David, J. <u>C.R. Acad. Sci. Paris.</u> 1955, 241, 116–118.
16. David, J. <u>Bull. Biol. France Belgique.</u> 1963, 97, 515–530.
17. Zacharuk, R.Y. <u>Can. J. Zool.</u> 1963, 41, 991–996.
18. Gangwere, S.K.; Chavin, W.; Evans, F.C. <u>Annal. of Entomol. Soc. Amer.</u> 1964, 57, 662–669.
19. Kolyer, J.M. <u>J. Res. Lep.</u> 1966, 5, 136–152.
20. Peters, T.M.; Chevone, B.I. <u>Mosquito News.</u> 1968, 28, 24–28.
21. Daum, R.J.; Gast, R.T.; Davitch, T.B. <u>J. Econ. Entomol.</u> 1969, 62, 943.
22. Hayes, D.K.; Schechter, M.S. <u>J. Econ. Entomol.</u> 1070, 63, 997.
23. Barbosa, P.; Peters, T.M. <u>J. Med. Entomol.</u> 1970, 7, 693–696.
24. Barbosa, P.; Peters, T.M. <u>Histochem. J.</u> 1971, 3, 71.
25. Hendricks, D.E. <u>J. Econ. Entomol.</u> 1971, 64, 1404.
26. Jones, R.L.; Harrell, E.A.; Snow, J.W. <u>J. Econ. Ent.</u> 1972, 65, 123–126.
27. Bridges, A.C.; Cocke, J.; Olson, J.K.; Mayer, R.T. <u>Mosquito News.</u> 1977, 37, 227.
28. Barbieri, A. <u>Riv. Malariol.</u> 1928, 7, 456–463.
29. Schildmacher, H. <u>Biol. Zentralbl.</u> 1950, 69, 468–477.
30. Ware, G.W. <u>Pesticides Theory and Application;</u> W.H. Freeman: San Francisco, 1978; p. 12.
31. Blum, H.F. <u>Photodynamic Action and Diseases Caused by Light;</u> Rheinhold: New York, 1941.
32. Spikes, J.D.; Straight, R. <u>Annu. Rev. Phys. Chem.</u> 1967, 18, 409–436.
33. Spikes, J.D.; Livingston, R. <u>Adv. Radiat. Biol.</u> 1969, 3, 29–121.
34. Grossweiner, L.I. <u>Photophysiology,</u> 1970, 5, 1–33.
35. Wilson, T.; Hastings, J.W. <u>Photophysiology,</u> 1970, 5, 49–95.
36. Krinsky, N.I. <u>Trends Biochem. Sci.</u> (Pers. Ed.), 1977, 2, 35–38.
37. Spikes, J.D. <u>In The Science of Photobiology;</u> Smith, K.C., Ed.; Plenum, New York, 1977; p. 87–112.
38. Yoho, T.P.; Butler, L.; Weaver, J.E. <u>J. Econ. Entomol.</u> 1971, 64, 972–973.
39. Yoho, T.P. Ph.D. Dissertation, West Virginia University, Morgantown, 1972.
40. Yoho, T.P.; Weaver, J.E.; Butler, L. <u>Environ. Entomol.</u> 1973, 2, 1092–1096.
41. Yoho, T.P.; Butler, L.; Weaver, J.E. <u>Environ. Entomol.</u> 1976, 5, 203–204.
42. Graham, K.; Wrangler, E.; Aasen, L.H. <u>Can. J. Zool.</u> 1972, 50, 1625–1629.
43. Broome, J.R.; Callaham, M.F.; Lewis, L.A.; Ladner, C.M.; Heitz, J.R. <u>Comp. Biochem. Physiol.</u> 1975, 51C, 117–121.
44. Broome, J.R.; Callaham, M.F.; Heitz, J.R. <u>Environ. Entomol.</u> 1975a, 4, 883–886.
45. Callaham, M.F.; Broome, J.R.; Lindig, O.H.; Heitz, J.R. <u>Environ. Entomol.</u> 1975, 4, 837–841.
46. Fondren, J.E., Jr.; Heitz, J.R. <u>Environ. Entomol.</u> 1978, 7, 843–846.
47. Fondren, J.E, Jr.; Norment, B.R.; Heitz, J.R. <u>Environ. Entomol.</u> 1978, 7, 205–208.
48. David, R.M.; Heitz, J.R. <u>J. Agr. Food Chem.</u> 1978, 26, 99–101.
49. Callaham, M.F.; Lewis, L.A.; Holloman, M.E.; Broome, J.R.; Heitz, J.R. <u>Comp. Biochem. Physiol.</u> 1975a, 51C, 123–128.

50. Callaham, M.F.; Palmertree, C.O.; Broome, J.R.; Heitz, J.R. Pest. Biochem. Physiol. 1977, 7, 21-27.
51. Weaver, J.E.; Butler, L.; Yoho, T.P. Environ. Entomol. 1976, 5, 840.
52. Weaver, J.E.; Butler, L.; Amrine, J.W., Jr. Environ. Entomol. 1982, 11, 463-466.
53. Broome, J.R.; Callaham, M.F.; Poe, W.E.; Heitz, J.R. Chem.-Biol. Interact. 1976, 14, 203-206.
54. Callaham, M.F.; Broome, J.R.; Poe, W.E.; Heitz, J.R. Environ. Entomol. 1977a, 6, 669-673.
55. Fondren, J.E., Jr.; Heitz, J.R. Environ. Entomol. 1978a, 7, 891-894.
56. Fondren, J.E., Jr.; Heitz, J.R. Environ. Entomol. 1979, 8, 432-436.
57. Lavialle, M.; Dumortier, B. C.R. Hebd. Seances Acad. Sci. 1978, 287, 875-878.
58. Clement, S.L.; Schmidt, R.S.; Szatmari-Goodman, G.; Levine, E. J. Econ. Entomol. 1980, 73, 390-392.
59. Creighton, C.S.; McFadden, T.L.; Schalk, J.M. J. Georgia Entomol. Soc. 1980, 15, 66-68.
60. Pimprikar, G.D.; Norment, B.R.; Heitz, J.R. Environ. Entomol. 1979, 9, 856-859.
61. Pimprikar, G.D.; Fondren, J.E., Jr.; Heitz, J.R. Environ. Entomol. 1980a, 9, 53-58.
62. Pimprikar, G.D.; Noe, B.L.; Norment, B.R.; Heitz, J.R. Environ. Entomol. 1980b, 9, 785-788.
63. Carpenter, T.L.; Heitz, J.R. Environ. Entomol. 1980, 9, 533-537.
64. Carpenter, T.L.; Heitz, J.R. Environ. Entomol. 1981, 10, 972-976.
65. Fairbrother, T.E.; Essig, H.W.; Combs, R.L.; Heitz, J.R. Environ. Entomol. 1981, 10, 506-510.
66. Carpenter, T.L.; Mundie, T.G.; Ross, J.H.; Heitz, J.R. Environ. Entomol. 1981, 10, 953-955.
67. Crounse, N.; Heitz, J.R. U.S. Patent 4 320 140, 1982.
68. Carpenter, T.L.; Johnson, L.H.; Mundie, T.G.; Heitz, J.R. J. Econ. Entomol. 1984, 77, 308-312.
69. Sakurai, H.; Heitz, J.R. Environ. Entomol. 1982, 11, 467-470.
70. Respicio, N. C.; Heitz, J.R. J. Econ. Entomol. 1983, 76, 1005-1008.
71. Respicio, N.C.; Heitz, J.R. J. Econ. Entomol. 1986, 79, 315-317.
72. Pimprikar, G.D.; Fondren, J.E., Jr.; Greer, D.S.; Heitz, J.R. Southwest. Entomol. 1984, 9, 218-222.
73. Pimprikar, G.D.; Heitz, J.R. J. Miss. Acad. Sci. 1984, 29, 77-80.
74. Carpenter, T.L.; Respicio, N.C.; Heitz, J.R. Environ. Entomol. 1984a, 13, 1366-1370.
75. Respicio, N.C.; Carpenter, T.L.; Heitz, J.R. J. Econ. Entomol. 1985, 78, 30-34.
76. Carpenter, T.L.; Respicio, N.C.; Heitz, J.R. J. Econ. Entomol. 1985, 78, 232-237.
77. Knox, J.P.: Dodge, A.D. Planta, 1985, 164, 22-29.
78. Knox, J.P.; Dodge, A.D. Planta, 1985a, 164, 30-34.

79. Robinson, J.R.; Beatson, E.P. Pest. Biochem. Physiol. 1985,
    24, 375-383.
80. Hawkins, J.A.; Healey, M.C.; Johnson-Delivorias, M.H.; Heitz,
    J.R. Veterin. Parasitol. 1984, 16, 35-41.
81. Berenbaum, M. J. Chem. Ecol. 1986, 12, 807-948.
82. Ivie, G.W. In Effects of Poisonous Plants on Livestock; Keeler,
    R., Van Kampen, K., James, L., Eds.; Academic: New York, 1978;
    pp. 475-485.
83. Berenbaum, M. Science, 1978, 201, 532-534.
84. Scott, B.R.; Pathak, M.A.; Mohn, G.R. Mutat. Res. 1976, 39,
    29-74.
85. Song, P.-S.; Tapley, K.J., Jr. Photochem. Photobiol. 1979,
    29, 1177-1197.
86. Berenbaum, M.; Feeny, P. Science, 1981, 212, 927-929.
87. Ivie, G.W.; Bull, D.L.; Beier, R.C.; Pryor, N.W.; Oertli, E.H.
    Science, 1983, 221, 374-376.
88. Bull, D.L.; Ivie, G.W.; Beier, R.C.; Pryor, N.W.; Oertli, E.H.
    J. Chem. Ecol. 1984, 10, 893-911.
89. Ivie, G.W.; Bull, D.L.; Beier, R.C.; Pryor, N.W. J. Chem Ecol.
    1986, 12, 869-882.
90. Ashwood-Smith, M.J.; Ring, R.A.; Liu, M.; Phillips, S.; Wilson,
    M. Can. J. Zool. 1984, 62, 1971-1976.
91. Bull, D.L.; Ivie, G.W.; Beier, R.C.; Pryor, N.W. J. Chem. Ecol.
    1986, 12, 883-890.
92. Uhlenbroek, J.H.; Bijloo, J.D. Rec. Trav. Chim. Pays-Bas Belg.
    1958, 77, 1004-1008.
93. Gommers, F.J. Nematologica, 1972, 18, 458-462.
94. Gommers, F.J.; Geerligs, J.W.G. Nematologica, 1973, 19, 389-393.
95. Bakker, J.; Gommers, F.J.; Nieuwenhuis, I.; Wynberg, H. J.
    Biol. Chem. 1979, 254, 1841-1844.
96. Gommers, F.J.; Bakker, J.; Smits, L. Nematologica, 1980, 26,
    369-375.
97. Wat, C.-K.; Prasad, S.K.; Graham, E.A.; Partington, S.; Arnason,
    T.; Towers, G.H.N. Biochem. Syst. and Ecol. 1981, 9, 59-62.
98. Arnason, T.; Swain, T.; Wat, C.-K.; Graham, E.A.; Partington,
    S.; Towers, G.H.N.; Lam, J. Biochem. Syst. and Ecol. 1981, 9,
    63-68.
99. Arnason, T.; Chan, G.F.Q.; Wat, C.K.; Downum, K.; Yamamoto, E.;
    Towers, G.H.N. Photochem. Photobiol. 1981a, 33, 821-824.
100. McLachlan, D.; Arnason, T.; Lam, J. Biochem. Syst. and Ecol.
     1986, 14, 17-23.
101. Arnason, T.; Swain, T.; Wat, C.K.; Graham, E.A.; Partington, S.;
     Tow, G.H.N.; Lam, J. Biochem. Syst. and Ecol. 1981b, 9,
     63-68.
102. Kagan, J.; Chan, G Experientia, 1983, 39, 402-403.
103. Kagan, J.; Prakash, I.; Dhawan, S.N.; Jaworski, J.A.
     Photobiochem. Photobiophys. 1984, 8, 25-33.
104. Kagan, J.; Kolyvas, C.P.; Lam, J. Experientia, 1984a, 40,
     1396-1397.
105. Downum, K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest. Biochem.
     Physiol. 1984, 22, 104-109.
106. Kagan, J.; Hasson, M.; Grynspan, F. Biochim. Biophys. Acta,
     1984b, 802, 442-447.

107. Reyftmann, J.P.; Kagan, J.; Santus, R.; Morliere, P. Photochem. Photobiol. 1985, 41, 1-7.
108. Kagan, J.; Kagan, E.D.; Siegneurie, E. Chemosphere, 1986, 15, 49-57.
109. Towers, G.H.N.; Arnason, J.T.; Wat, C.K.; Lambert, J.D.H. Can Pat. 1,173,743, 1984.
110. Horsley, C.H.J. Pharmacol. 1934, 50, 310-322.
111. Brockmann, H.H. Prog. Org. Chem. 1952, 1, 64-82.
112. Brockmann, H.H. Proc. Chem. Soc. London, 1957, 304-312.
113. Yamazaki, S.; Okube, A.; Akiyama, Y.; Fuwa, K. Agricult. Biol. Chem. 1975, 39, 287-288.
114. Macri, F.; Vianello, A. Plant Cell and Environ. 1979, 2, 267-271.
115. Daub, M.E. Phytopathology, 1982, 72, 370-374.
116. Daub, M.E. Plant Physiol. 1982a, 69, 1361-1364.
117. Daub, M.E.; Briggs, S.P. Plant Physiol. 1983, 71, 763-766.
118. Daub, M.E.; Hangarter, R.P. Plant Physiol. 1983, 73, 855-857.
119. Knox, J.P; Dodge, A.D. Plant Cell and Environ. 1985b, 8, 19-25.
120. Ellsworth, R.K.; Aronoff, S. Arch. Biochem. Biophys. 1969, 130, 374-383.
121. Rebeiz, C.A. Chemtech. 1982, 12, 52-63.
122. Rebeiz, C.A.; Hopen, H.J. PCT Int. Appl. WO 8, 600, 785.
123. Maltotsy, A.G.; Fabian, G. Nature, 1946, 149, 877.
124. Maltotsy, A.G.; Fabian, G. Arch. Biol. Hungarica, 1947, 17, 165-170.
125. Kagan, J.; Kagan, E. Chemosphere, 1986a, 15, 243-251.
126. Kagan, J.; Kagan, E.D.; Kagan, I.A.; Kagan, P.A.; Quigley, S. Chemosphere, 1985, 14, 1829-34.
127. Kagan, J.; Kolyvas, C.P.; Jaworski, J.A.; Kagan, E.D.; Kagan, I.A.; Zang, L.-H. Photochem. Photobiol. 1984c, 40, 479-483.
128. Molero, M.L.; Hazen, M.J.; Stockert, J.C. J. Plant Physiol. 1985, 120, 91-94.

Chapter 2

Type I and Type II Mechanisms
of Photodynamic Action

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Mechanisms of photooxidation of organic compounds are discussed, and methods of determining photooxidation mechanisms reviewed. Two cases that have been particularly well studied, cercosporin and α-terthienyl, are used to exemplify the techniques.

Many chemicals, including natural cell constituents, can absorb light and photosensitize damage to organisms. Some of these compounds are used by organisms (including man) to attack or defend against other organisms. This process, called "photodynamic action", requires oxygen and damages biological target molecules by photosensitized oxidation. Biochemical effects include enzyme deactivation (through destruction of specific amino acids, particularly methionine, histidine, and tryptophan), nucleic acid oxidation (primarily of guanine), and membrane damage (by oxidation of unsaturated fatty acids and cholesterol) (1, 2).

Mechanisms of Photooxygenation

Photosensitized oxidations are initiated by absorption of light by a sensitizer, which can be a dye or pigment, a ketone or quinone, an aromatic molecule, or many other types of compound. The sensitizer (**Sens**) is converted to an electronically excited state by absorption of a photon. The initial product is a short-lived singlet (^{1}**Sens**); in many cases, this undergoes intersystem crossing to the longer-lived triplet (^{3}**Sens**). Because the singlet generally has a very short lifetime, only reactants at relatively high concentration can interact with it before it decays; however, much lower concentrations are sufficient to react with the longer-lived triplet state.

$$\text{Sens} \xrightarrow{\text{hv}} {}^1\text{Sens} \longrightarrow {}^3\text{Sens}$$

There are two mechanisms of photosensitized oxidation, named "Type I" and "Type II" by Gollnick (3) (see Fig. 1). In the Type I process, substrate or solvent reacts with the sensitizer excited state (either singlet or triplet, **Sens***) to give radicals or radical ions, respectively, by hydrogen atom or electron transfer. Reaction of these radicals with oxygen gives oxygenated products. In the Type II process, the excited sensitizer reacts with oxygen to form singlet molecular oxygen ($^1O_2$), which then reacts with substrate to form the products. The Type I and Type II mechanisms are always in competition; factors which govern the competition include oxygen concentration, the reactivities of the substrate and of the sensitizer excited state, the substrate concentration, and the singlet oxygen lifetime (4). These factors will be discussed in more detail in a subsequent section.
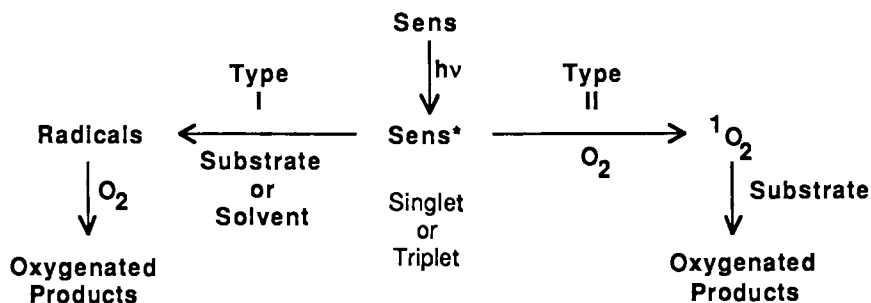

Fig. 1. Mechanisms of Photosensitized Oxidation

Type I Processes

Electron transfer both to and from molecules takes place more readily in the excited state than in the ground state (5,6). This follows from the fact that an electron is promoted from a strongly binding orbital to one that is less strongly binding on going from the ground state to the excited state. This process results in the production of a reducing electron and an oxidizing hole in the excited state, as shown in Fig. 2.

A well-studied example of the electron-transfer Type I process is the oxidation of aromatic olefins (Donor), by electron-poor aromatics such as dicyanoanthracene (DCA), which results in transfer of an electron to the singlet excited state of the aromatic from the olefin (7). The resulting radical anion of the sensitizer is immediately reoxidized by oxygen, producing a superoxide ion-aromatic radical cation pair. These react to give the observed products, mainly oxidative cleavage of the olefins.
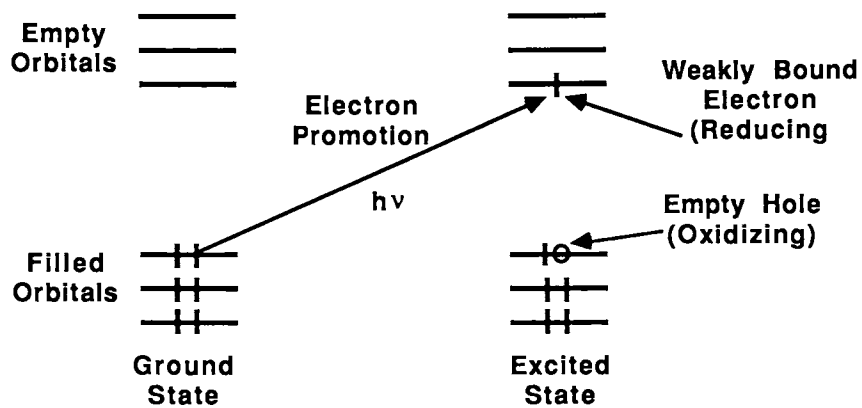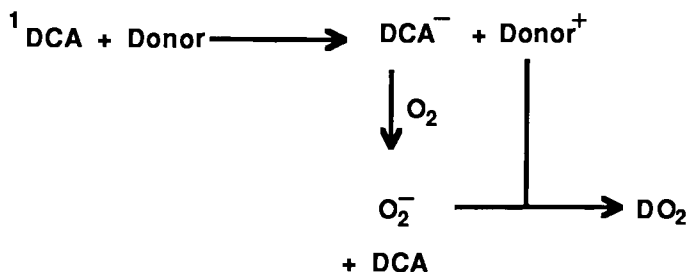
Fig. 2. Electron Promotion In Excited State

$$^{1}DCA + Donor \longrightarrow DCA^{-} + Donor^{+}$$

$$\downarrow O_2$$

$$O_2^{-} \longrightarrow DO_2$$

$$+ DCA$$

The Type I reaction can also result in hydrogen abstraction, giving radical products. These radicals can react directly with oxygen to give peroxides or initiate radical chain autoxidation. Hydrogen abstraction is particularly common with ketone and quinone sensitizers, but also occurs with many dyes, although usually less efficiently. Substrates that are good hydrogen donors promote this reaction (4).

$$R_2C=O^* + R'-H \longrightarrow R_2\overset{.}{C}\text{-OH} + R'\cdot$$

$$\downarrow O_2$$

Radical Chain Reactions $\longleftarrow$ R'OOH
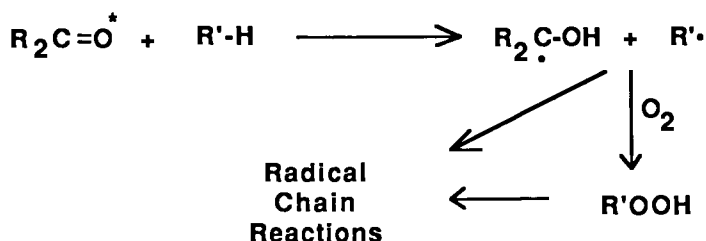
The Type II Process

The Type II reaction produces singlet molecular oxygen, which reacts directly with substrates to give oxygenated products or decays to the ground state if it fails to react. The rate of decay is strongly dependent on solvent: in water, the lifetime of singlet oxygen is about four microseconds, while in organic solvents (and presumably also in the lipid regions of membranes), the lifetime is on the order of ten to twenty times longer (8,9).

$$^{3}O_2 \longleftarrow {}^{1}O_2 \xrightarrow[\text{Substrate}]{} \text{Substrate} \cdot O_2$$
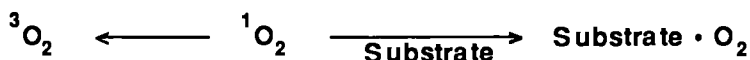
Two major classes of singlet oxygen reactions are additions to olefins with allylic hydrogens, giving allylic hydroperoxides, with a shift in position of the double bond (the "ene" reaction, 10), and addition to dienes, aromatics, and heterocycles, giving endoperoxides (the Diels-Alder reaction, 11).

Other processes include reaction of electron-rich olefins to give unstable four-membered ring peroxides called dioxetanes (12), oxidation of sulfides to sulfoxides (via an intermediate "persulfoxide", 13), and reaction of electron-rich phenols, including tocopherol, to unstable hydroperoxy-dienones (14).

$$^1O_2 \; + \; R_2S \; \longrightarrow \; R_2S^+OO^- \; \longrightarrow \; 2\,R_2S^+O^-$$
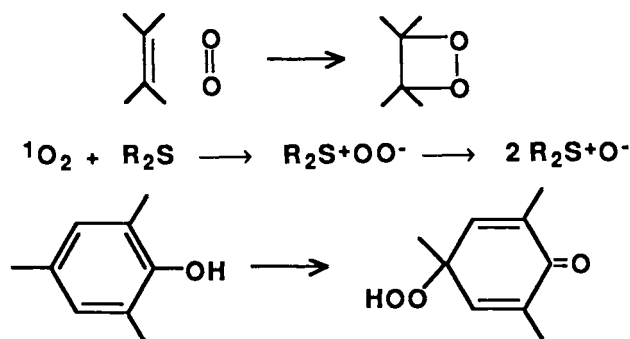
Many compounds deactivate (i. e., quench) singlet oxygen efficiently without reacting (15). For example, β-carotene inhibits photooxidation of many compounds efficiently at even very low concentrations by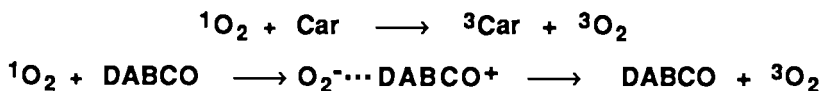 an energy transfer mechanism, without being appreciably oxidized itself. Other compounds such as DABCO (1,4-diazabicyclooctane) and azide ion quench singlet oxygen by a charge-transfer process. Phenols and sulfides also quench singlet oxygen, in competition with their oxidation. For example, α-tocopherol quenches singlet oxygen at a high rate in all solvents, but reacts rapidly only in protic solvents (16-18).

$$^1O_2 \; + \; Car \; \longrightarrow \; ^3Car \; + \; ^3O_2$$

$$^1O_2 \; + \; DABCO \; \longrightarrow \; O_2^{-}\cdots DABCO^+ \; \longrightarrow \; DABCO \; + \; ^3O_2$$

Singlet oxygen is an electronically excited molecule, and can return to the ground state with emission of light (19). There are two types of singlet oxygen luminescence, from a single molecule at 1.27 μm, and "dimol" lumin-escence at 634 and 704 nm. Both types of luminescence are very inefficient because the lifetime of singlet oxygen in solution is short compared to the radiative lifetime. Because the dimol emission depends on a bimolecular collision between two short-lived species, its efficiency also depends on the concentration of singlet oxygen.

$$^1O_2 \; \longrightarrow \; h\nu \; (1.27\mu) \qquad 2\,(^1O_2) \; \longrightarrow \; h\nu \quad (634,704nm)$$
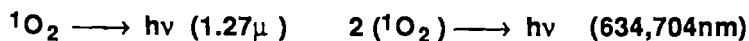
Determination of Mechanism

As mentioned in the introduction, high sensitizer reactivity, high substrate reactivity, and concentration, low oxygen concentration, and short singlet oxygen lifetimes favor the Type I mechanism, while the opposite factors favor the Type II. One of the most direct methods of determining whether a reaction is proceeding via a Type I or a Type II mechanism is to vary substrate and oxygen concentration and determine the amount of products formed under various conditions. This technique is particularly useful in homogeneous solution, especially where there are distinct sets of products from the two mechanisms. At sufficiently high oxygen and/or low substrate concentration, a reaction can be forced into a clean Type II pathway, whereas the Type I pathway can be forced under the opposite conditions. Changing solvent to one in which the singlet oxygen lifetime is longer helps to favor the Type II mechanism. Binding of sensitizer to substrate (e.g., membrane, protein, or nucleic acid) is particularly common in living organisms, and tends to favor Type I mechanisms because of the effective increase in substrate concentration (20,21).

There are two examples where the competition between Type I and Type II mechanisms has been particularly well documented, 1,1-diphenylmethoxyethylene (DPME, 22) and dimethyl stilbene (23). In both cases, the reaction can be manipulated by means of the factors described above to give dioxetane products via the electron-transfer pathway or Diels-Alder or ene products, respectively, via the Type II route.
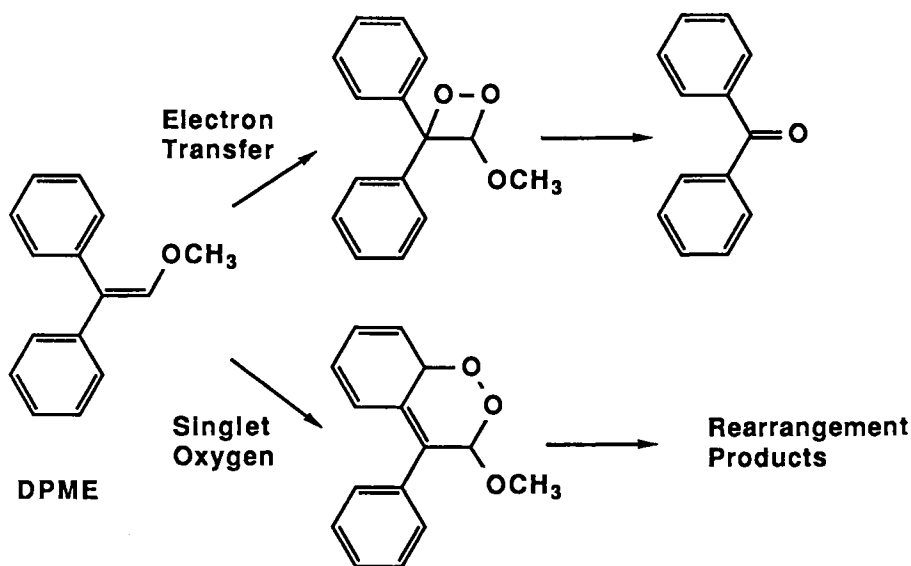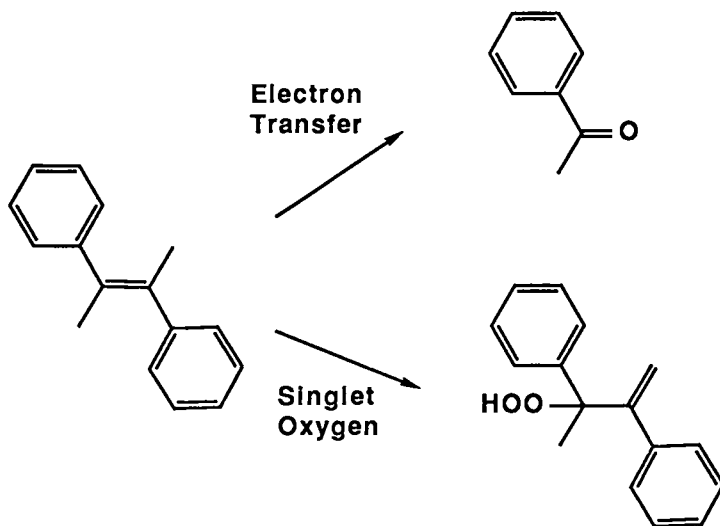
Establishing the mechanism of photosensitized oxidations in complex systems is a difficult task (24-26). Kinetic tests and the use of inhibitors for various reactive species are more ambiguous than in homogeneous solution, because reagents are often compartmentalized, bound, or localized, and it is rarely possible to know the local concentrations of various reacting species, sensitizers, quenchers, and traps.

Many workers have used allegedly specific traps or quenchers for various reactive species, including singlet oxygen, superoxide ion (O_2^-), hydroxyl radical (OH·), peroxy radicals (ROO·), and other oxidants. However, the specificity of traps and inhibitors for oxidants requires far more study than it has received. For instance, all reagents and quenchers for singlet oxygen have low oxidation potentials and will also interact with other oxidants. Also, almost all quenchers of singlet oxygen can quench sensitizer excited states as well. Quenching of sensitizer excited states can be distinguished from singlet oxygen quenching by determining the degree of inhibition at several oxygen concentrations, since if singlet oxygen is being quenched, the degree of inhibition will not depend on the oxygen concentration.

Interconversions and interactions among reactive species complicate the process further. In both Type I and Type II reactions, the initial products are often peroxides, which can break down to induce free radical reactions. Such secondary thermal reactions have been shown to cause much of the photodynamic damage observed in membranes under some conditions (27,28). Radical chains can cause the oxidation of many molecules of

starting material for each primary product. For this reason, product analysis may reflect mainly secondary chain processes rather than the primary reaction mechanism. Similar comments apply to inhibition studies.

Methods of assessing the relative importance of various processes are needed. Detection of an intermediate is a necessary but not sufficient condition for its having a causative role in a process. It does little good to show that a reactive intermediate is present without being able to estimate what fraction of the overall oxidation it causes. Such quantitation has rarely been accomplished in heterogeneous systems.

Techniques for Characterizing Singlet Oxygen

A large number of techniques have been developed for detection of possible reactive intermediates in biological oxygen damage (24, 29). For reasons of space, this report will concentrate on techniques that are useful for the detection and characterization of singlet oxygen.

Chemical Traps. A large number of compounds have been added to reacting systems as traps for singlet oxygen, and the formation of the supposedly characteristic products used as an indication of the intermediacy of $^1O_2$. For example, dimethylfuran reacts with singlet oxygen to give the diketone shown below as the ultimate product. Unfortunately, so do a very large number of other oxidants. In fact, furans are a prime example of very nonspecific singlet oxygen traps (24).
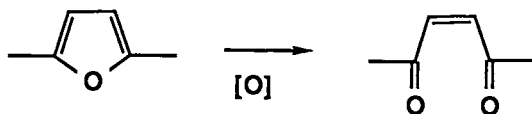
A diagnostic trap for singlet oxygen is cholesterol, which reacts with singlet oxygen to give the 5-α hydroperoxide; reactions with radical and other oxidants give complex mixtures, but the 5-α product is not among them (30). This system is somewhat limited because of the low reactivity of cholesterol with singlet oxygen. Although cholesterol is not soluble in water, it can be bound to microspheres, allowing its use in aqueous systems (31).
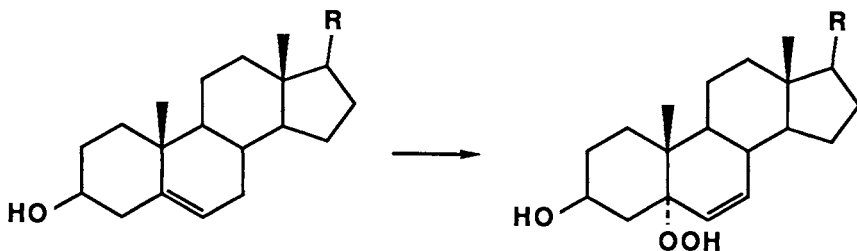
A second trapping system which also appears to be specific uses suitably substituted anthracenes (32, 33). Anthracene derivatives are considerably more reactive than cholesterol. These compounds can be made soluble in any medium by suitable choice of substituents. One drawback to this system is that anthracenes are also photosensitizers, so that when small amounts of product are formed, adventitious photooxidation must be carefully ruled out.
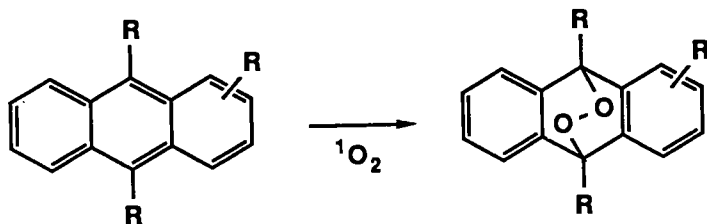
A third trapping system makes use of the fact that polyunsaturated fatty acids react with singlet oxygen to give a mixture of conjugated and unconjugated isomers of the product hydroperoxides, whereas only the conjugated isomers are formed on radical attack (34). The unconjugated products thus serve as characteristic singlet oxygen fingerprints. This system, like the cholesterol trap, is somewhat difficult to use, since the isomers must be separated by HPLC.
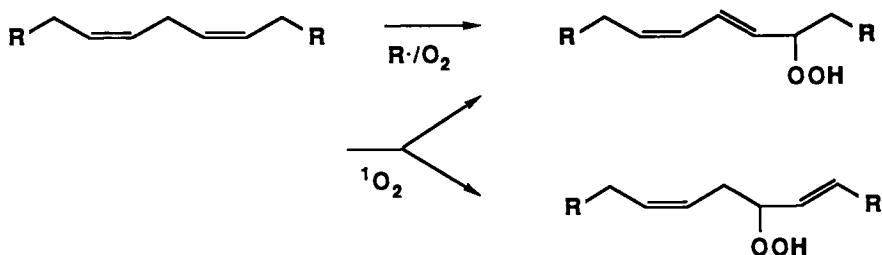
A further system is suggested by Cadet, who has isolated the hydroxylactam shown below from photooxidation of guanosine, and has shown that this compound can be used as a fingerprint for the presence of singlet oxygen (35). This compound is probably the product of rearrangement of the initial peroxide, which is not stable at room temperature.
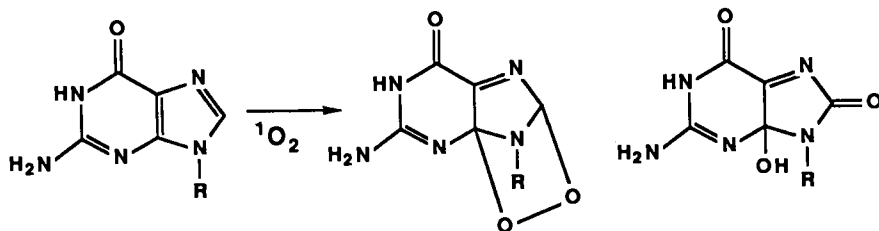
Two other trapping systems are used primarily for kinetic characterization of singlet oxygen; neither is likely to be useful in systems where there is more than one strong oxidant. One is a sensitive system using the production and ESR detection of the nitroxide radical from a tertiary amine (a process whose mechanism and stoichiometry are poorly understood) (36). The second uses the bleaching of a p-nitrosodimethylaniline on reaction with the peroxide produced by singlet oxygen and histidine as a measure of singlet oxygen production (37).

Inhibitors. As mentioned above, many compounds such as carotene, DABCO, and azide, are effective quenchers for singlet oxygen. These compounds, and others which react with singlet oxygen, are frequently used to inhibit reactions in which singlet oxygen is thought to be a reactive intermediate. Care must be taken in interpretation of the results, however, because of their lack of specificity, as discussed above. One way of using inhibitors that partly avoids this problem is to use a quantitative treatment, calculating the amount of singlet oxygen expected to be inhibited from known rate constants and comparing it with that observed (24). The quantitative kinetic technique cannot be used in inhomogeneous solutions, where the local concentration of the inhibitor cannot be calculated.

D_2O Effect. Singlet oxygen has a longer lifetime in D_2O than in H_2O (9, 38). Thus many reactions of singlet oxygen proceed more efficiently in D_2O than in H_2O. However, there are two important limitations to this technique. First, singlet oxygen reactions in the two solvents will differ in efficiency only if solvent quenching of singlet oxygen limits its lifetime; if substrate or quencher is already removing all the $^1O_2$, there will be no effect of deuteration on the lifetime. Secondly, it has been shown that O_2^- also has a longer lifetime in D_2O than in H_2O (39), and reactions of superoxide ion would therefore also be expected to be more efficient in the deuterated solvent. The effect of solvent deuteration on other possible reactive species has not been shown. Thus, this effect cannot be used to distinguish between reactions of $^1O_2$ and O_2^-.

"Clean" Sources of Singlet Oxygen. One useful technique for studying suspected singlet oxygen reactions is to generate singlet oxygen under carefully defined conditions free of any other reactive species, and compare its effects with those of the suspect system. Photochemical systems (using unreactive sensitizers, at high O_2 pressure, and with low concentrations of substrates that are unreactive in the Type I reaction) can often be used. Another technique is to use a reverse Diels-Alder reaction, using a naphthalene endoperoxide (40); this technique can be used under very mild conditions (37 °C, neutral), and no side reactions have yet been reported. Most other known chemical sources of singlet oxygen (e. g., hypochlorite/H_2O_2, phosphite ozonides (41)) involve very strong oxidants which can react with singlet oxygen substrates.
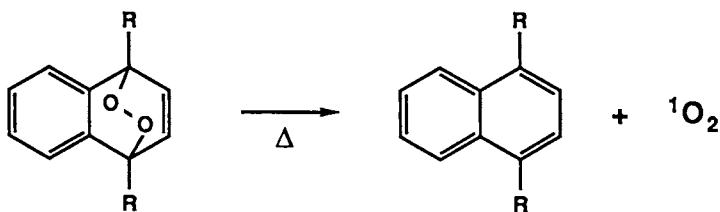
Luminescence. Dimol (visible) luminescence may be specific for singlet oxygen, if the wavelength is carefully measured (42), but can not be easily used to determine the amount of singlet oxygen present, since it depends on a second-order reaction between two singlet oxygen molecules. It is essential that the wavelength of the emission be carefully determined; in many cases, the source of light emission was subsequently found to be something other than singlet oxygen when the wavelength was determined. Dimol emission is also difficult to interpret because the extreme sensitivity of photomultipliers allows the measurement of tiny amounts of light that may have little relationship to the major chemical processes going on. The infrared luminescence of singlet oxygen can be quantitatively related to the amount of singlet oxygen produced, and can lend confidence to its identification if the wavelength is carefully established (43, 44).

A short pulse of laser light can be used to excite singlet oxygen sensitizers, and the resulting intensity and decay rate of the $1.27\mu m$ luminescence of singlet oxygen can be detected by a germanium photodiode with a low-noise amplifier and a digitizer with signal averaging; a schematic of the apparatus is shown in Fig. 3 (9, 45, 46). The amount of singlet oxygen produced and its lifetime can be measured very easily this way. This technique provides a definitive and quantitative method of characterizing singlet oxygen produced in photochemical systems. Furthermore, by measuring the change of lifetime of $^1O_2$ when a reagent is added, the rate of its reaction with $^1O_2$ can be simply and rapidly determined.

The yield of singlet oxygen photosensitized by photodynamic sensitizers can be measured using this apparatus. The intensity of the $^1O_2$ lumin-escence is compared with that of a sensitizer of known singlet oxygen yield under conditions where the two sensitizers have equal optical density. These values are checked by measuring the amount of a well-characterized singlet oxygen substrate photolyzed in a given time. With correction for the inefficiency of singlet oxygen trapping (which can be calculated from the known rate of reaction of the substrate and the decay rate of singlet oxygen in the solvent), the amount of singlet oxygen produced in a given time can be calculated. This value can converted to a quantum yield by measuring the number of quanta absorbed from the lamp in a given time by conventional actinometry.

The infrared luminescence determination measures the loss of singlet oxygen and nothing else, so that it is possible to measure absolute rates of singlet oxygen reactions with biological acceptors with confidence and

simplicity. The sensitivity and time response must be optimized for the time-resolved system to be usable in aqueous media, where the lifetime of singlet oxygen is much shorter than in organic solvents.

Transient Absorption Spectroscopy. Transient absorption spectro - scopy is useful for measuring measuring the absorption of both radical ions and triplet molecules on a nanosecond time scale (47, 48). The sensitizer is excited by a short pulse of light, usually from a laser, and the absorbance of the transient species measured by a lamp / photodetector system, shown schematically in Fig. 4. This apparatus is useful for observing transient intermediates from photodynamic sensitizers or acceptors undergoing Type I reaction if either the reduced sensitizer or the oxidized acceptor has a measurable absorbance, as most do.

Conductivity. Time-resolved conductivity measurements have not previously been used much in this field, but should be very useful for the study of electron-transfer Type I mechanisms. The apparatus shown in Fig. 5 is widely used in pulse radiolysis (49). For photochemical work, the sensitizer is excited by a pulsed light source, and the change in conductivity measured as a function of time. This apparatus can be used on a microsecond or nanosecond time scale by slight modifications. The sensitivity for detection of ions is excellent, in fact better than that of optical techniques.

Examples

Two examples of mechanistic studies on photodynamic pesticides that have been studied in unusual detail will be presented to illustrate the uses of some of the techniques described in this article.

Cercosporin. The fungal pigment cercosporin, the structure of which is shown below, acts photodynamically on plant tissues, causing electrolyte leakage and other damage; these effects probably aid the attack of the fungus on the plant (50,51). This pigment causes lipid peroxidation in the presence of light and oxygen, and the action spectrum for the damaging effects is the same as the absorption spectrum of cercosporin (52).
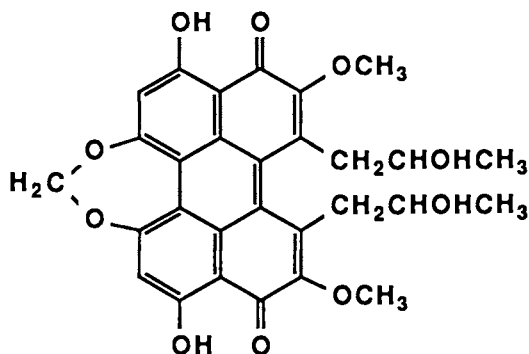
Fig. 3. Singlet Oxygen Detection

Fig. 4. Transient Absorption Spectroscopy

Fig. 5. Conductivity Apparatus

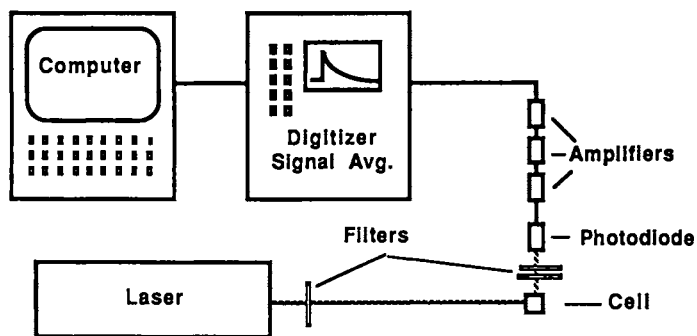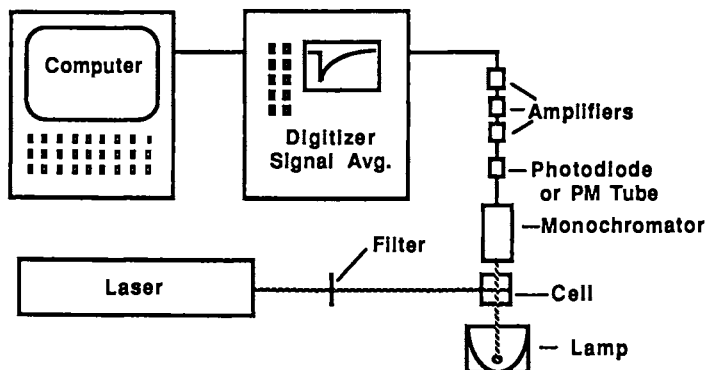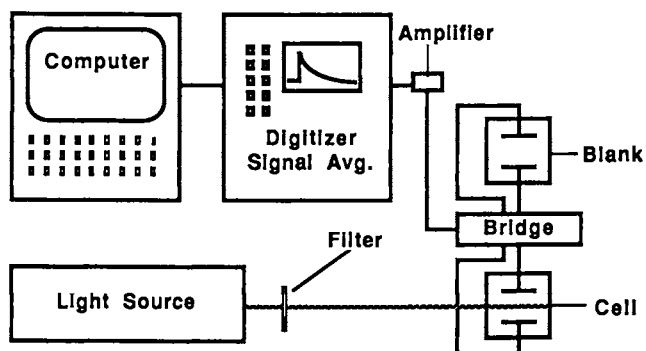The products of photooxidation of oleic and linoleic acids and of cholesterol sensitized by this pigment were identical to those with singlet oxidation (53, 54). The oxidation is inhibited by carotenoids and DABCO (52). Damage is also inhibited by various phenolic antioxidants (53), but this may be caused by inhibition of radical chain autoxidation of the lipids by breakdown of the initial peroxides.

The characteristic 1.27 μm singlet oxygen emission is readily observed when cercosporin solutions in C_6D_6 are irradiated (55). The quantum yield of singlet oxygen is 0.81, as determined by comparison with meso-porphyrin IX dimethyl ester. This value was confirmed by 2-methyl-2-pentene photo - oxidation.

Terthienyl. α–Terthienyl (α–T) is a member of a class of photodynamic sensitizers, the polyacetylenes, which are present in a number of plant species (56). The plants apparently use these compounds as to protect against insect attack; after ingesting the materials, insects or their ova are killed photodynamically. (However, the nematocidal activity observed with this compound is difficult to explain on the basis of a photodynamic mechanism, because no appreciable light would be expected to penetrate the soil to the depth of the nematodes.)

α–T has been shown to kill a wide variety of cells, and, although there has been some disagreement on this score, there is a requirement for oxygen (57, 58). The action spectrum for the damaging effects is the same as the absorption spectrum of α–T.
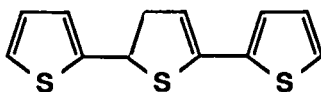
The mechanism of action of this compound has been reviewed (57). There is considerable chemical evidence that singlet oxygen is produced by α–T on irradiation with near-UV light. Inhibition of the effects by inhibitors of other reactive oxygen species is not observed, but a variety of singlet oxygen quenchers protect against deactivation of enzymes by this compound, and there is a positive D_2O effect on the deactivation of enzymes. The dioxetane, a typical singlet oxygen product, can be formed by α–T-sensitized photooxidation of adamantylideneadamantane. Differences between the biological activities of α–T and the singlet oxygen sensitizer methylene blue have been observed, but they may be due to differences in localization between the lipophilic α–T and the polar methylene blue.

The fluorescence yield of α–T in various solvents is less than 0.1, and the triplet yield is substantial, on the order of 0.2. The singlet oxygen yield in ethanol was reported to be between 0.15 and 0.2 (58).

Singlet oxygen production by α–T is observed by 1.27 μm emission (R. Kanner and C. S. Foote, in preparation). The quantum yield of singlet oxygen production is high in benzene, as established by comparison of the luminescence yield with that of several sensitizers with known quantum

yields of singlet oxygen production. We are currently attempting to determine this quantum yield more precisely, but present results suggest the yield is around 0.8.   It s not certain why our results differ from those of Kagan, Santus et al; the solvent is different, and these authors used a somewhat indirect method of determining the quantum yield of singlet oxygen formation, the disappearance of diphenylisobenzofuran. A very recent paper has reported the singlet lifetime of α-T to be very short, and has characterized the photophysical properties of both the singlet and triplet (59).

Summary

Production of singlet oxygen from these both cercosporin and α-T has been unequivocally demonstrated. Since in both cases, the physiological effects of the photodynamic action of both compounds have been shown to be inhibited by singlet oxygen quenchers, both necessary and sufficient conditions for the intermediacy of singlet oxygen in the action of these compounds appear to be present.

Acknowledgments

Literature Cited

1.   Straight,   R. C. ; Spikes, J. D. In Singlet O_2;   Frimer, A. A. Ed. ; CRC : Boca Raton, Fla. 1985; Vol. IV, 85-144.
2.   Elstner, E. F. Ann. Rev. Plant Physiol. 1982, 33, 73-96.
3.   Gollnick, K. Advan. Photochem. 1968, 6, 1-122.
4.   Foote, C. S. Free Radicals in Biology 1976, 2, 85-133.
5.   Mattes, S. L. ; Farid, S. Science 1984, 226, 917-21.
6.   Mattes, S. L. ; Farid, S. In Organic Photochemistry; Padwa, A. Ed.; Marcel Dekker: New York , 1983; 233-326.
7.   Foote, C. S. Tetrahedron 1985, 41, 2221-7.
8.   Wilkinson, F. ; Brummer, J. G. J. Phys Chem. Ref. Dat. 1981, 10, 809-1000.
9.   Monroe, B. In Singlet O_2; Frimer, A. A. Ed. ; CRC: Boca Raton, Fla. 1985; Vol. I, pp 177-224.
10. Gollnick, K. ; Kuhn, H. J. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York, 1979; pp 287-429.
11. Frimer, A. A. In The Chemistry of Peroxides. Patai, S. , Ed. ; J. Wiley and Sons:, New York , 1983; Chapter 7.
12. Bartlett, P. D. ; Landis, M. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York , 1979; pp 244-86.
13. Ando, W. ; Takata, T. In Singlet O_2; Frimer, A. A. Ed. ; CRC: Boca Raton, Fla. 1985; Vol. III, pp 1-118.

14. Saito, I. ; Matsuura, T. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York , 1979; pp 511-74.
15. Foote, C. S. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York, 1979; pp 139-73.
16. Foote, C. S. ; Ching, T. - Y. ; Geller, G. G. Photochem. Photobiol. 1974, 20, 511-4.
17. Stevens, B. ; Small, R. D. ; Perez, S. R. Photochem. Photobiol. 1974, 20, 515-8.
18. Fahrenholtz, S. R. ; Doleiden, F. H. ; Trozzolo, A. M. ; Lamola, A. A. Photochem. Photobiol. 1974, 20, 505-9.
19. Kasha, M. ; Brabham, D. T. In Singlet Oxygen; Wasserman, H. H.; Murray, R. W. , Eds. ; Academic: New York, 1979; pp 1-34.
20. Valenzeno, D. P. Photochem. Photobiol. 1983, 37S, 105-.
21. Bellin, J. S. ; Grossman, L. I. Photochem. Photobiol. 1965, 4, 45-53.
22. Steichen, D. S. ; Foote, C. S. J. Am. Chem. Soc. 1981, 103, 1855-7.
23. Gollnick, K. ; Schnatterer, A. Photochem. Photobiol. 1986, 43, 365-78.
24. Foote, C. S. In Biochemical and Clinical Aspects of Oxygen; Caughey, W. S. , Ed. ; Academic: New York, 1979; pp 603-26.
25. Krinsky, N. I. In Oxygen Radicals in Chemistry and Biology; Bors, W. , Saran, M. ; Tait, D. , Eds. , DeGruyter: Berlin, 1984; 453-64.
26. Krinsky, N. I. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York , 1979; pp 597-642.
27. Lamola, A. A. ; Yamane, T. ; Trozzolo, A. M. Science 1973, 179, 1131-3.
28. Doleiden, F. H. ; Fahrenholtz, S. R. ; Lamola, A. A. ; Trozzolo, A. M. Photochem. Photobiol. 1974, 20, 519-21.
29. Singh, A. Can. J. Physiol. Pharm. 1982, 60, 1330-45.
30. Kulig, M. J. ; Smith, L. L. J. Org. Chem. 1973, 38, 3639-42.
31. Foote, C. S. ; Shook, F. C. ; Abakerli, R. A. Meth. Enzymol. 1984, 105, 36-47.
32. Schaap, A. P. ; Thayer, A. L. ; Faler, G. R. ; Goda, K. ; Kimura, T. J. Am. Chem. Soc. 1974, 96, 4025-6.
33. Lindig, B. A. ; Rodgers, M. A. J. ; Schaap, A. P. J. Am. Chem. Soc. 1980, 102, 5590-3.
34. Thomas, M. ; Pryor, W. Lipids 1980, 15, 544-8.
35. Cadet, J. ; Decarroz, C. ; Wang, S. Y. ; Midden, W. R. Isr. J. Chem. 1983, 23, 420-9.
36. Lion, Y. ; Delmelle, M. ; Van De Vorst, A. Nature 1976, 263, 442-3.
37. Kralic, I. ; El Mohsni, S. Photochem. Photobiol. 1978, 28, 577-81.
38. Kearns, D. R. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W., Eds. ; Academic: New York, 1979; pp 115-38.
39. Bielski, B. H. J. ; Saito, E. J. Phys. Chem. 1971, 75, 2263-6.
40. Saito, I. ; Matsuura, T. ; Inoue, K. J. Am. Chem. Soc. 1983, 105, 3200-6.
41. Murray, R. W. In Singlet Oxygen; Wasserman, H. H. ; Murray, R. W. , Eds. ; Academic: New York, 1979; pp 59-114.

42. Deneke, C. F. ; Krinsky, N. I. Photochem. Photobiol. 1977, 25, 299-304.
43. Kanofsky, J. Biochem. Biophys. Res. Comm. 1986, 134, 777-82.
44. Keene, J. P. ; Kessel, D. ; Land, E. J. ; Redmond, R. W. ; Truscott, T. G. Photochem. Photobiol. 1986, 43, 117-20.
45. Ogilby, P. R. ; Foote, C. S. J. Am. Chem. Soc. 1983, 105, 3423-30.
46. Hurst, J. R. ; Schuster, G. B. J. Am. Chem. Soc. 1983, 105, 5756-60.
47. Weir, D. ; Scaiano, J. C. ; Arnason, J. T. ; Evans, C. Photochem. Photobiol. 1985, 42, 223-30.
48. Malba, V. ; Jones, G. E. II, Poliakoff, E. D. Photochem. Photobiol. 1985, 42, 451-5.
49. Asmus, K. D. Meth. Enzymol. 1984, 105, 167-78.
50. Cavallini, L. ; Bindole, A. ; Macri, F. ; Vianello, A. Chem. Biol. Interact. 1979, 28, 139-46.
51. Daub, M. E. Plant Physiol. 1982, 69, 1361-4.
52. Daub, M. E. Phytopathology 1982, 72, 370-4.
53. Youngman, R. J. ; Schieberle, P. ; Schnabl, H. ; Grosch, W. ; Elstner, E. F. Photobiochem. Photobiophys. 1983, 6, 109-19.
54. Daub, M. ; Hangartner, R. P. Plant Physiol. 1983, 73, 855-7.
55. Dobrowolski, D. C. : Foote, C. S. Angew. Chem. 1983, 95, 729-30.
56. Arnason, T. ; Towers, G. H. N. ; Philogene, B. J. R. ; Lambert, J. D. H. Am. Chem. Soc. Symposium Ser. 1983, 208, 139-51.
57. Cooper, G. K. ; Nitsche, C. I. Bioorg. Chem. 1985, 13, 362-74.
58. Reyftmann, J. P. ; Kagan, J. ; Santus, R. ; Morliere, P. Photochem. Photobiol. 1985, 41, 1-7.
59. Evans, C. ; Weir, D. ; Scaiano, J. C. ; Mac Eachern, A. ; Arnason, J. T. ; Morand, P. ; Hollebone, B. ; Leitch, L. C. ; Philogene, B. J. R. Photochem. Photobiol. 1986, 44, 441-51.

Chapter 3

Photomodification and Singlet Oxygen Generation in Membranes

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Photomodification of cell membranes, which in many cases is critical for cell killing, is governed by the properties of membrane associated sensitizer. The heterogeneous structure of biological membranes can be an important factor in photosensitization reactions. Sensitizers and protective agents may associate preferentially with the hydrophobic membrane core, may accumulate at the aqueous interface or may bind to membrane proteins. Such localization effects can alter photomodification rates. Although singlet oxygen can diffuse across membrane interfaces in high yield in some cases, membrane associated sensitizer mediates most membrane photomodifications. The membrane environment can influence singlet oxygen generation. Model studies have shown that singlet oxygen quantum yields increase with decreasing solvent polarity. In liposomes or micelles both quantum yields and lifetimes are increased. Aggregation states of sensitizers are changed in the membrane environment leading to alteration of singlet oxygen production. Finally increases in temperature can increase singlet oxygen production due to effects on membrane fluidity.

The goal of this chapter is to describe the characteristic features of singlet oxygen generation in membranes as they are currently understood. Membrane photomodification has been singled out for special consideration for two major reasons. First recent years have seen an explosion of interest in membrane phenomena as the scientific community has become aware that cellular membranes are much more than mere gossamer bags that hold the inside in and the outside out. Second in the instances where cellular, tissue and organism photomodification has been examined in detail cell membranes have repeatedly been identified as critical targets of modification (1-7).

The Membrane Environment

Biological membranes. Not only are membranes critical cellular
components and critical targets for photomodification, they also
present a unique environment for photosensitizers which generate
singlet oxygen. In fact if this were not the case we would not need
to consider membrane sensitization as a separate topic. The
characteristics of singlet oxygen generation by sensitizers in
aqueous solution would apply. As we shall see this is not the case.
Membranes present an environment that differs from the surrounding
medium not only in polarity, water content and dielectric constant,
but they are heterogenous structures which present a variety of
domains with which sensitizers can associate and from which they can
act.

Current concepts of the structure of cell membranes are based on
the fluid mosaic model of Singer and Nicolson (8). In this view,
Figure 1A, the membrane phospholipids are arranged in a fluid
bilayer. Their hydrophobic, hydrocarbon tails are oriented toward
the center of the bilayer exposing their polar head groups to the
aqueous environment at either surface. This arrangement is
stabilized by the hydrophobic forces between the phospholipids and
does not involve covalent bonding. The majority of the phospholipids
are thus free to diffuse within the plane of the membrane, but move
with difficulty from one surface of the bilayer to the other.
Membrane proteins are inserted into the lipid bilayer, either part
way or entirely spanning the bilayer (so called integral or intrinsic
proteins). The portions of these proteins which are in contact with
the hydrophobic interior of the bilayer are composed of a high
proportion of hydrophobic amino acids, while the portions exposed at
the aqueous interface have a high proportion of hydrophilic amino
acids. Thus the proteins are also stabilized in position by
hydrophobic forces and have the same ability to diffuse in the plane
of the bilayer but not across it. Both the proteins and
phospholipids can have carbohydrate groups attached to them, but such
groups have been found only at the outside surface of the cell. Most
animal cell membranes have a variable content of cholesterol
interspersed with the phospholipids. The proportion of cholesterol
is especially high in the membrane of the red blood cell, which is
the membrane studied most extensively.

The most significant modification of these ideas that has
occurred in recent years has been the discovery that in many
instances integral membrane proteins are restricted in their motion
by an intracellular skeleton of peripheral (or extrinsic) membrane
proteins that serve to anchor some of the intrinsic proteins in
loosely fixed positions. In the red cell the cytoskeletal network of
peripheral membrane proteins lies just below the membrane surface
and anchors integral proteins, which span the bilayer, at periodic
points via a protein component known as ankyrin (9,10).

The result of the membrane structure just described is that the
interior of the membrane has the characteristics of the interior of a
lipid bilayer. The dielectric constant (polarity) is very low (2-3)
in this region. Lipophilic solutes can be expected to partition
readily into this domain. Water is present in greatly reduced
concentration with some investigators claiming that the bilayer is

devoid of water.   Water permeability of most membranes is, however,
quite high.
     The other significant feature of the membrane environment   which
deserves   comment   is   the   interfacial region.   This   is   a   region
extending   roughly   from the glycerol backbone of   the   phospholipids
away   from   the   membrane to the end   of   the   attached   carbohydrate
moieties. This region is of intermediate polarity between the bilayer
interior   and   the aqueous environment (dielectric constant of   about
10).   Due to the polar nature of the charged groups at the   membrane
interface,   the   surface   of   most   biological membranes has   a   net
negative charge.   This surface charge can modify the distribution of
charged solutes near it.   In particular the concentration of cations
is   higher   and   the concentration of anions is lower   within   a   few
angstroms   of the surface than in the bulk solution adjacent   to   the
membrane.   In transport studies it has even been possible to discern
the effects of local surface charge, i.e. charged groups located near
the   opening   of a proteinaceous channel through the   membrane   (11).
Water   present   near   the interface is thought to be aligned   by   the
charged groups into a structure more like ice than liquid water.

Membrane   model   systems.   Model systems have been very   valuable   as
guides   to   understanding the characteristics of   singlet   oxygen   in
membrane   systems.   However,   the   results must also be verified   in
biological membranes since the assembly of phospholipids and proteins
of   a   cell membrane is significantly more complex   than   most   model
systems.   Still,   in   many   instances   models   provide   the   only
information currently available.
     The model systems most often employed are micelles or liposomes,
(Figure 1B).   The former are aqueous dispersions of amphipathic
molecules.   These molecules which have a hydrophobic and hydrophilic
portion   spontaneously form aggregates in aqueous solution such   that
the interior of the aggregate,   or micelle,   contains the hydrophobic
portions and thus mimics the membrane interior.   The area of contact
with water mimics the membrane interfacial region and can be   charged
of   either   sign,   or   uncharged depending on the   structure   of   the
amphipathic molecule used.   Liposomes are membranous structures which
resemble soap bubbles.   Many amphipathic molecules will spontaneously
form   such structures when agitated with an aqueous phase.   They can
be either unilamellar,   that is composed of a single bilayer with   an
enclosed   aqueous   phase,   or   multi-lamellar,   in   which   there   are
multiple   bilayers   enclosing the aqueous   phase.   The   incorporated
aqueous   phase   can have a different composition from the   suspension
medium.

Sensitizer – Membrane Interactions

The   ability of sensitizers to generate singlet oxygen   in   membranes
can   be   influenced   by interaction of the sensitizer   with   membrane
components.   Binding   of sensitizer to substrate has been   shown   to
favor   Type   I reactions in homogenous   solutions.   Modification   in
which the sensitizer is physically separated from the target suggests
a   Type II reaction.   The sensitizers to be considered here   include
the   halogenated fluorescein derivatives (xanthenes),   the porphyrins
and   merocyanine-540.   These were selected because they   are   widely
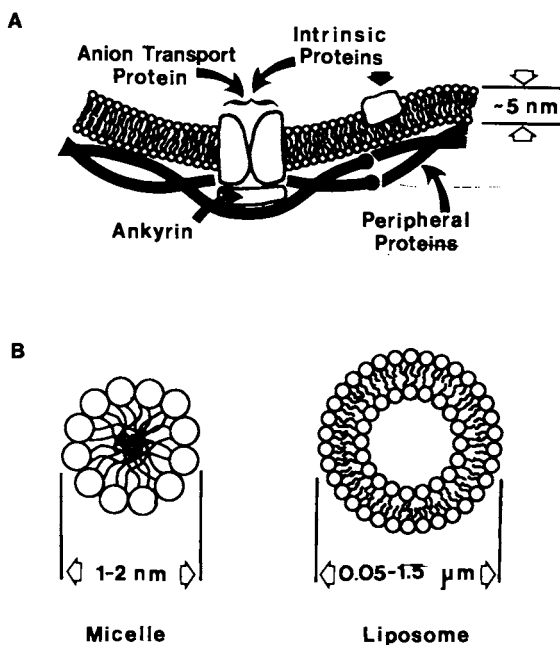
Figure 1. Membrane Structure. (A) The red blood cell membrane.
Dimensions are approximate. (B) Two model membrane systems.
Phospholipids with their two hydrocarbon chains are depicted as
circles with two wavy lines.

studied singlet oxygen sensitizers which affect cell membranes, but
in some instances exhibit widely different behavior.

Sensitizer External to the Membrane. The simplest sensitizer –
membrane interaction is the absence of interaction. That is, the
sensitizer generates singlet oxygen in the aqueous medium external to
the membrane which then diffuses to the membrane and effects a
modification. Within its 3 to 4 microsecond lifetime in aqueous
solution (12-14) singlet oxygen can diffuse about 0.1 microns before
it decays to the ground state (15,16). Since this distance is
considerably greater than the 0.005 micron thickness of biological
membranes, modification by singlet oxygen generated external to the
membrane is a distinct possibility. Experimental verification has
been found in the work of Bezman, et al. (17) who demonstrated that
bacteria could be photoinactivated by singlet oxygen generated by
rose bengal immobilized on large polystyrene beads. In model systems
also singlet oxygen has been shown to penetrate into micelles from
the aqueous suspension medium (18). Liposomes can even be modified
by singlet oxygen generated in the gas phase and then bubbled into
the suspension medium (19). [See section D.5. for penetration of
singlet oxygen through membranes.] However, with very few
exceptions, photosensitization in biological systems occurs with
membrane association of the sensitizer, either by dissolution of the
sensitizer in the hydrophobic membrane interior or by binding to some
membrane component (4, 20-22).

Sensitizer Bound to the Membrane. In instances where a
photosensitizer is attached to a ligand with known binding properties
the localization of sensitizer with respect to the membrane is fairly
well defined. In an attempt to localize the critical target for
photomodification of erythrocyte membranes Pooler and Girotti (23)
used Eosin Y attached to an isothiocyanate moiety, a compound which
is known to bind specifically to the membrane protein responsible for
anion transport (and possibly transport of other materials). While
the photochemical properties and singlet oxygen generating capability
of Eosin-isothiocyanate were not altered from free Eosin Y, it was 50
to 100 times as effective as a sensitizer for photohemolysis. Thus
the site of generation of the singlet oxygen was crucial. These
results have been interpreted as an implication for a critical role
of the dimeric red cell anion transport protein in photohemolysis
(23,24). Since the production of membrane lesions leading to
photohemolysis occurs through the combined action of two photons and
two sensitizer molecules (22, 25-27), a dimeric protein is a likely
target.
      Another example of the exploitation of known binding properties
arises for the sensitizer merocyanine-540 (M-540). M-540 was used as
an optical probe of membrane potential in the 70's (28) in excitable
cells. It was subsequently shown that M-540 has an affinity for
excitable cells and hematopoietic cells (29, 30), but of even more
interest was the demonstration that most of this binding is of
relatively low affinity whereas binding to leukemic cells is of very
high affinity (31). In a manner similar to the treatment of
malignancies with hematoporphyrin derivative and light, M-540 and
light is now being developed for the treatment of leukemia. In
aqueous solution M-540 generates singlet oxygen upon illumination

(32). It binds to the surface of cell membranes (33) and preliminary evidence suggests that singlet oxygen is involved in its photomodification of membranes (Valenzeno, et al., unpublished results).

Sensitizer Associated with the Membrane. Membrane associated sensitizer is more important for most photosensitization reactions than sensitizer dissolved in the bathing medium. Bagchi and Basu (34) were able to demonstrate that acriflavine molecules remaining outside E. coli were ineffective at photoinactivation by simply diluting the external medium just before illumination. Photoinactivation proceeded at a rate governed by the concentration of sensitizer in which the bacteria were incubated, not the concentration in which they were subsequently illuminated.

The commonly used halogenated fluorescein sensitizers, which include both eosin Y and rose bengal, also produce photomodification while associated with membranes. The action spectra for photomodification of lobster axon membranes by four sensitizers of this class show red shifts when compared to the absorption spectra in aqueous solution (35). An alteration of the environment of the sensitizer as would occur upon association with the membrane can explain the red shift. The localization of the sensitizer within the membrane has not been well studied. Evidence must be gleaned from studies in a variety of fields. Varnadore, et al., (36) have measured photo-voltages produced by erythrosin B, a halogenated fluorescein, across bilayer membranes. Their results suggest that erythrosin B localizes at the plane of the glycerol region of the membrane phospholipids. Other halogenated fluoresceins, although not studied as thoroughly, yielded similar photo-voltages. This is consistent with oxidation-reduction reactions mediated by erythrosin B in brain membranes (37). In micelles both rose bengal and erythrosin B localize near the interface (38-39) as do merocyanines (40). Using merocyanine-540 Lelkes and Miller (33) have shown a localization near the glycerol region of cell membranes.

The relative potency of ten different halogenated fluorescein sensitizers for membrane photomodification varies over a range of 5,000 to 35,000-fold for red cells and nerve cells respectively (21,22). Yet the photochemical properties and relative effectiveness for enzyme inactivation in aqueous solution differ by only a factor of about 20 (41). The discrepancy can again be attributed to sensitizer localization and differing photochemical properties in the membrane environment. In the case of cell membranes the relative effectiveness of the halogenated fluoresceins was accounted for by assuming that sensitizer partitions between the aqueous suspension medium and the membrane, and then absorbs light and creates singlet oxygen in that environment. The results of modeling based on these assumptions are consistent with the observed variation in potency, Figure 2. This variation cannot be accounted for if it is assumed that sensitizer acts from the suspension medium. These results suggest that sensitizer photochemical properties and ability to generate singlet oxygen may be different in membranes. We will consider this in some detail below.

Association of sensitizer with membranes, assessed as sensitizer hydrophobicity, has been shown to be a critical determinant of sensitizing efficacy for porphyrins as well as halogenated
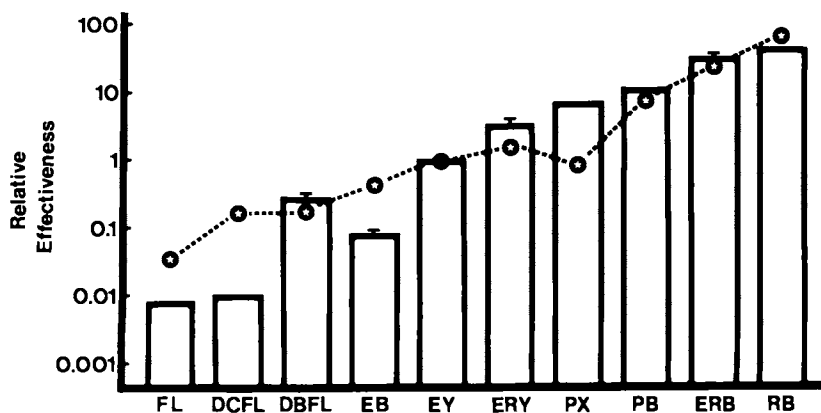
Figure 2. Relative effectiveness of halogenated fluoresceins. The
bars represent the measured values of relative effectiveness for
photohemolysis of the sensitizers listed. The points are the pre-
dicted effectiveness values based on a model that assumes that the
sensitizer acts from the membrane environment, not the aqueous sus-
pension medium. FL = fluorescein; PX = tetrabromodichlorofluorescein;
all other abbreviations are defined in Figure 3. Adapted from Ref. 22.

fluoresceins (2, 42,43). Mesoporphyrin accumulates in a region with a dielectric constant of about 20 in L1210 cells from which it is an active sensitizer (44). Such a dielectric constant suggests an intramembranous localization near the interface as was the case for the fluorescein derivatives. Suwa, et al. (45) demonstrated that hematoporphyrin sensitized the oxidation of membrane cholesterol much more efficiently when it was incorporated into the membrane phase of liposomes than when it was dissolved in the aqueous suspension medium. Additionally, it has been shown that the most hydrophobic fractions of the tumor sensitizer mixture, known as hematoporphyrin derivative, which partition best into membranes are the most effective sensitizers (46, 47). Action spectra for both hematoporphyrin and its active fraction lead to the suggestion that these sensitizers are bound to membrane proteins during photoexcitation (46).

Finally, we should note that both binding and partitioning may be important for membrane association of sensitizers. The photosensitizing potency of halogenated fluoresceins, as described above, is proportional to their ability to partition between aqueous medium and membrane-like lipid solvents. Recent work, however, has shown that the final distribution of sensitizer does not follow a simple partitioning isotherm, but involves binding of the sensitizer to membrane sites (26, 27). Similar conclusions based on completely different experimental evidence have been drawn for some porphyrins (46).

Characteristics of Singlet Oxygen in Membranes

Biochemistry of Singlet Oxygen Modification of Membranes.

Singlet oxygen is capable of modifying many components of biological membranes. John Spikes will discuss the biochemistry of photodynamic action in Chapter 6, but a brief discussion is in order here so that the mechanisms of singlet detection in membranes will be understandable.

Unsaturated bonds in phospholipids are susceptible to attack by singlet oxygen leading to a variety of peroxidized lipids. Malonaldehyde, a product of polyunsaturated fatty acid oxidation, is readily detectable using a simple colorimetric method (48). Side chains of five amino acids are photomodifiable. These include histidine, tyrosine, tryptophan, cysteine and methionine. More extensive modification can produce protein cross-linking which is more easily detected by gel electrophoresis. Finally cholesterol can be modified by singlet oxygen to produce the characteristic 5 alpha hydroperoxide of cholesterol. Only singlet oxygen is known to produce this oxidation product of cholesterol. For a more detailed discussion of singlet oxygen chemistry, see Chapter 2 by Christopher Foote.

Determination of the intermediacy of singlet oxygen in membrane modifications follows the methods available in solution. Typically a quencher such as azide or a reactant such as a furan are used to compete with solvent quenching and reaction with membrane-located substrate. A reduction in modification suggests a singlet oxygen mechanism. Conversely, an increase in modification when deuterium oxide replaces water (which quenches singlet oxygen) indicates a singlet oxygen reaction. [Deuterium oxide is known to increase

singlet oxygen lifetimes compared to aqueous solution about 15-fold
(13, 49)].   In   membrane   systems,   however,   there   is   the   added
complexity of access of the modifying agent to the membrane.   Some
quenchers/reactants (beta-carotene and alpha-tocopherol) are very
lipid soluble and partition readily into membranes. Others (azide,
imidazole) are not likely to penetrate into the hydrocarbon core of
the bilayer.   Deuterium oxide effects may also be severly reduced
since the reactions may not be occurring in an aqueous environment.
For   these   reasons   it   is   frequently   observed   that   higher
concentrations of quenchers, reactants and/or deuterium oxide are
required for an observable effect in membrane systems (15, 50-52).

Singlet Oxygen Quantum Yields The quantum yield of singlet oxygen
formation (number of singlet oxygen molecules generated per absorbed
photon) is frequently found to be higher in membranes than in aqueous
media for porphyrins, while current evidence suggests the opposite
for fluorescein sensitizers, Figure 3.   Blum and Grossweiner (53)
showed that in the presence of small unilamellar liposomes and
porphyrins singlet oxygen quantum yields were increased compared to
the results in phosphate buffer.   They attributed this effect to
solubilization   and   monomerization   of   their   sensitizers
(hematoporphyrin   and   the active fraction   of   hematoporphyrin
derivative) by the liposomes. The quantum yields for hematoporphyrin
were doubled while they were increased about 15-fold for the active
fraction of hematoporphyrin derivative.   The quantitative results of
this study must be viewed with caution, however, since it is based on
the assumption that singlet oxygen quantum yields sensitized by rose
bengal   are   the   same   in aqueous suspension as in membranes (see
below).   Such a decrease in singlet quantum yields due to aggregation
of sensitizer has been found in other studies under a variety of
solvent/model membrane conditions (14, 54, 55).   In a study by Reddi,
et al., (56) it was noted that triplet quantum yields of the
sensitizer in micelles were similar to those found in organic
solvents for both coproporphyrin and hematoporphyrin, both being
elevated from the values in aqueous media. However, the ability of a
sensitizer triplet to generate singlet oxygen was about twice as
large in the organic solvent as in either the micelles or aqueous
buffer.   The reasons for this solvent effect on singlet oxygen
generation are not clear, but cannot be attributed to micellar
surface charge or organic solvent polarity since the results were
invariant when these parameters were changed.   Similar observations
of solvent-dependent increases in singlet oxygen quantum yields, in
excess of that which can be explained by aggregation effects have
been attributed to chemical effects on the porphyrin side chains
(14).   This last study provides an interesting demonstration of the
importance   of   the   solvent   for   singlet   quantum   yields.
Hematoporphyrin   was   dissolved   into   the   aqueous   phase   of   an
octanol/water system and was allowed to distribute between the two
phases.   A narrow beam of light was used to illuminate a small area
of solution and the 1.27 micron luminescence produced by singlet
oxygen was monitored.   When the exciting beam was moved across the
phase boundary from water to octanol a marked increase in
luminescence was detected indicating a higher singlet oxygen
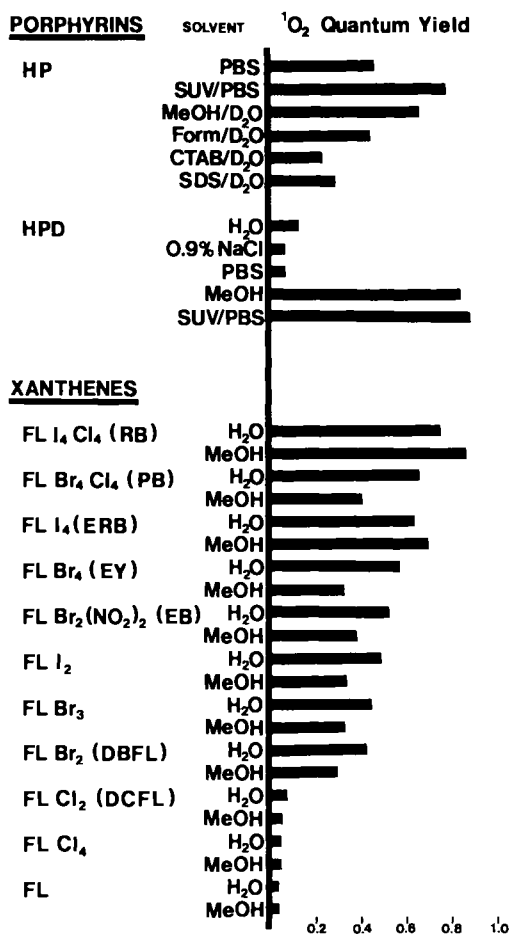concentration.

Figure 3. Singlet oxygen quantum yields of selected porphyrins and halogenated fluoresceins (xanthenes). Singlet quantum yields are plotted in various solvent systems as available in the literature. Adapted from Refs. 53, 54, 56, and 58.

With fluorescein sensitizers triplet quantum yields, singlet oxygen quantum yields and sensitizing effectiveness all increase with the degree of halogenation of the sensitizer (21, 22, 57, 58). The triplet quantum yield increase is due to the heavy atom effect of halogenation leading to an increase in intersystem crossing from the excited singlet state of the sensitizer to the excited triplet (59). Increases in singlet oxygen yield and sensitizing effectiveness follow from increased triplet yield.

Studies with fluoresceins using organic solvents to mimic membranes have generally shown decreases in sensitizer triplet and singlet oxygen quantum yields in these solvents. In contrast to the porphyrins, fluoresceins do not aggregate in the micromolar to millimolar range (58). Thus there is no aggregation effect on the yields. Rather shifts in the electronic energy levels with solvent become important (58). These lead to a reduction in triplet sensitizer quantum yields and singlet oxygen quantum yields in media less polar than water. Gandin, et al. (57) have shown that both yields are lower in aerated ethanol than in aqueous solutions. Based on their literature review, these investigators assumed that all sensitizer triplets are quenched by generation of singlet oxygen in aerated ethanol but that singlet oxygen is produced in only about 70% of the triplet quenchings in aqueous solutions. A similar pattern of decreased triplet quantum yield in a solvent of lower polarity, but with concommitant dimerization, has been demonstrated for chlorophylls a and b by Bowers and Porter (60).

Singlet Oxygen Lifetimes. In addition to the total yield of singlet oxygen its lifetime will influence the degree of reaction. The longer oxygen remains in an excited state the more likely it is to modify a given target. The membrane environment can affect singlet oxygen lifetimes and thus alter the reaction probability and mean diffusion distance of the excited state. For example in a hydrocarbon environment like the interior of a membrane Pooler and Valenzeno (15) calculated that the length constant (distance at which the concentration of singlet oxygen has fallen to 1/e of its concentration at its source) for singlet oxygen diffusion was increased about 2-1/2-fold over that in aqueous solution. This means that 37% of the singlet oxygen generated will be able to diffuse about 0.25 micrometers before losing its energy of excitation.

Since there are no experimentally determined values for singlet oxygen lifetime in biological membranes the results of model studies provide the only information, Figure 4. In the organic solvent, formamide, singlet oxygen lifetime is reported to be increased compared to the 3 to 4 microsecond lifetime in aqueous solvents (61). In micelles singlet lifetimes are increased to varying degrees. In both anionic and cationic micelles singlet lifetimes have been shown to be increased to 20-25 microseconds (62). These values were the same as those found in the corresponding pure hydrocarbon lacking the charged head group. Thus in this case charged groups at the interface were unimportant. On the other hand Lindig and Rodgers (12) reported that in the presence of deuterium oxide, which prolongs singlet oxygen lifetimes, the interfacial region could affect singlet lifetimes. With either anionic or cationic micelles in deuterium oxide singlet lifetime was found to be 54 microseconds (i.e. about
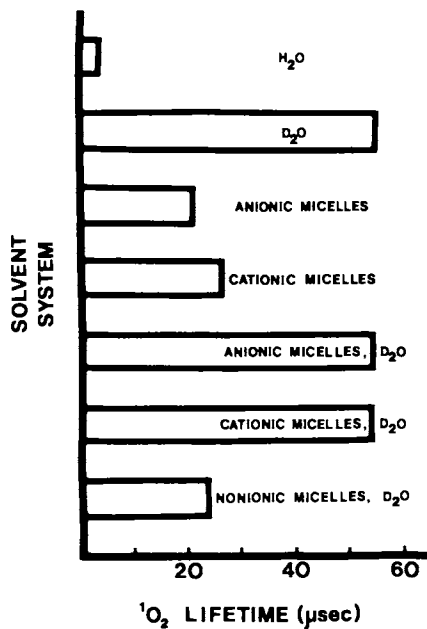
Figure 4. Singlet oxygen lifetimes in different solvent systems.
Adapted from Refs. 12, 13, 14, and 62.

13-fold longer than in water). With nonionic micelles the lifetime was only 21 to 26 microseconds. The reduction was attributed to quenching by hydroxyl groups at the interfacial region of the nonionic micelles. A second study confirmed that some, but not all, non-ionic micelles produce similar lifetime reductions in deuterium oxide (49).

The functional groups at the interface may not be the only way in which singlet lifetimes are affected by this boundary. Based on studies in a reverse micellar system (aqueous aggregates in a hydrophobic solvent), Miyoshi and Tomita (63) have proposed that singlet oxygen may not be subject to quenching by structured water near membrane surfaces. Thus there would be no effect of deuterium oxide in this domain. Azide quenching of and tryptophan reactivity with singlet oxygen also appear to be reduced in this domain (64). The issue of the interaction of singlet oxygen with the membrane interface raises an important question. How well can singlet oxygen diffuse into, out of, or through membranes?

Singlet Oxygen Diffusion across Membranes. Oxygen diffuses very easily through membranes and all biological membranes are quite permeable to oxygen. There is no reason to suspect that excited state oxygen should differ from the ground state in its diffusibility. In fact all the calculations of mean diffusion distances for singlet oxygen use the diffusion coefficient for ground state oxygen. Oxygen solubility is actually greater in hydrocarbon solvents and micelles than in water (62). On the other hand it is not obvious that singlet oxygen, in traversing a membrane, will remain in the excited state. Not only is there a change in medium polarity upon entering the hydrocarbon core, but the various functional groups associated with the interfacial region must be penetrated.

It is now known that singlet oxygen can penetrate membranes under appropriate conditions. Gorman, Lovering and Rodgers (18) attacked this problem by making SDS micelles containing a reagent, diphenyl isobenzofuran (DPBF), which is bleached specifically by singlet oxygen. To this suspension they added a sensitizer, methylene blue, which is confined to the aqueous suspension medium. Upon irradiation the DPBF was bleached by singlet oxygen which diffused from the medium into the micelles. They further showed that if they produced a second set of micelles containing the lipid-soluble sensitizer, pyrene, and added these to micelles containing DPBF, singlet oxygen could diffuse out of the pyrene containing micelles where it was generated and into the DPBF micelles to produce bleaching, Figure 5. Of particular note is the finding that in the methylene blue system the rate of DPBF bleaching by singlet oxygen was reduced 50% by the compartmentalization. Thus singlet oxygen was able to penetrate into the micelles but an appreciable fraction may have been quenched. Many studies have since been performed using micelles, reversed micelles and liposomes. Some studies have shown essentially no hindrance in singlet oxygen penetration of the membrane model (65). Others have claimed no hindrance but have actually shown a variable decrease in reaction rate (66 {5-22% inhibition},67 {12.5%}). However, a few studies have shown significant interaction of the membrane with penetrating singlet
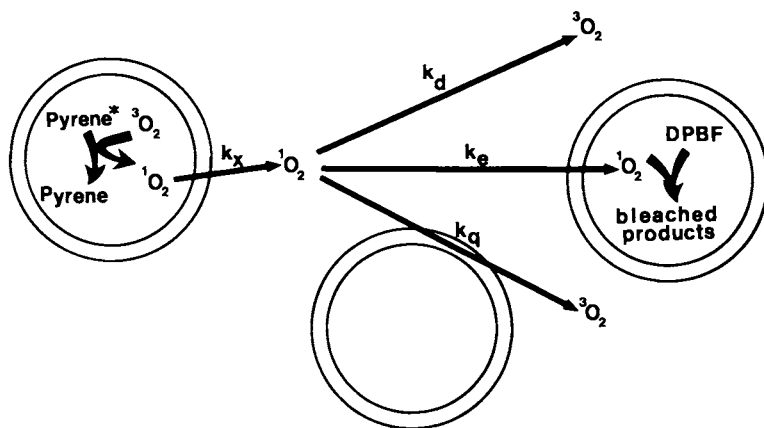
Figure 5. Diagram of singlet oxygen reactions in a micellar system.
Singlet oxygen generated by photoexcited pyrene can diffuse out of
the micelle in which it was produced. Three competing pathways
exist with differing rate constants. Spontaneous deexcitation to
the ground state, k_d, quenching by empty micelles, k_q, and entry
into a DPBF-containing micelle, k_e. Bleaching of DPBF by singlet
oxygen is followed spectrophotometrically. Adapted from Refs. 18
and 63.

oxygen. Miyoshi and Tomita (68) found that micelles produced quenching of singlet oxygen as efficiently as azide. They estimated that the probability of singlet oxygen penetration given an encounter with a micelle was about 0.38 to 0.48. Gorman, et al. (18) estimated this value as only 0.1. Suwa, et al. (45) demonstrated that cholesterol incorporated into micelles was efficiently photomodified only if sensitizer was also in the micelle, not if it was dissolved in the suspension medium. Finally Jori and co-workers (69) found that sensitizer separated in micellar solution from its target was able to modify it only under some conditions. How can these diverse results be reconciled? Certainly the differences in micelle or liposome composition, sensitizer employed and target can influence the results. This is reflected by the results described above in which singlet oxygen lifetimes were reduced by neutral but not cationic or anionic micelles in a single study (12). The conclusion seems to be that under some conditions in simple model systems singlet oxygen may penetrate membranes easily, but in other other instances there can be significant quenching of the penetrating oxygen. The situation is reminiscent of the issue of effective sensitizer location. Although sensitizer external to the membrane may effect modification through singlet oxygen generation, in biological systems membrane associated sensitizer is the effective species. So, here also, the real question is what is the penetrability of singlet oxygen for biological membranes? All of the model systems discussed are devoid of proteins. Membrane proteins are good targets for reaction with singlet oxygen. Thus, significant reduction in singlet oxygen concentrations may occur as it passes into or through protein-containing biological membranes. No experimental evidence is available concerning this point.

Effects of Temperature and Membrane Fluidity. Temperature effects on membrane photomodification appear to be diverse at first sight. For photohemolysis by fluorescein derivatives Blum, et al., (70) showed almost no temperature dependence for the photomodification process (during illumination) and Davson and Ponder (71) showed that even the photodynamic lysis occuring after light was relatively independent of temperature. Blum and Kauzmann (72) were able to show that at severely reduced temperatures, $-79°$ and $-210°$ C, photohemolytic membrane modification was greatly reduced and abolished respectively. On the other hand sensitizer association with the membrane varies directly with temperature in the interval before illumination (Pooler, personal communication). In yeast cells photoinactivation sensitized by toluidene blue is accelerated at higher temperatures with a break point at $21-22°$ C (73). This has been attributed to a change in membrane fluidity at the transition temperature of the membrane.

Membranes have been shown to alter their dye permeability at the phase transition of the membrane lipids (74). In liposomes also there appears to be a difference in photomodification rate which is dependent on the phase transition of the lipid. Suwa, et al. (75) used two different lipids with different transition temperatures. Photomodification of cholesterol incorporated into the liposomes was greatly increased above the respective transition temperature of each type of liposome. The enhanced photomodification was associated with

an enhanced uptake of the sensitizer, hematoporphyrin. High
cholesterol levels are known to abolish phase transitions. With high
cholesterol levels (1:2, cholesterol:phospholipid) hematoporphyrin
incorporation and cholesterol modification showed no abrupt
alteration with temperature.

The above findings are most consistent with a temperature
dependence of sensitizer association with the membrane. In most
studies in which temperature was not varied until the time of
illumination or after, no temperature dependence was seen. When pre-
illumination incubation occured at different temperatures the
temperature dependence was detected. Suwa, et al. (75), while
recognizing the importance of sensitizer association proposed that
this could not entirely account for the observed temperature
dependence. They felt that in addition to facilitating sensitizer
association, the increase in mèmbrane fluidity with temperature
augmented photomodification rates by enhancing oxygen solubility.
This was based on the findings of Kimmich and Peters who reported
that oxygen solubility is increased about 3-fold above the phase
transition temperature of lecithin bilayers. Since many biological
membranes do not exhibit well defined phase transitions the
applicability of this observation to cell membranes is uncertain.

Summary and Conclusions

Membrane photomodification and singlet oxygen generation in membranes
are obviously different from the analogous processes in simple
homogenous solution. Membranes are structured, compartmentalized
systems of lipids, proteins and cholesterol with domains of varying
hydrophobicity and reactivity. The interaction of sensitizers with
the membrane can be pivotal in sensitization reactions. Both
halogenated fluoresceins and porphyrins appear to localize near the
membrane interface and are effective from that location. They are
relatively ineffective, for modification of biological membranes,
when generating singlet oxygen in the medium external to the
membrane.

Singlet oxygen can modify many membrane components. Singlet
oxygen quantum yields may be either increased or decreased in the
membrane environment depending on the sensitizer employed.
Porphyrins are disaggregated by membrane association and demonstrate
increased quantum yields. Halogenated fluoresceins, which show no
aggregation effects, have lower quantum yields in membranes. Singlet
oxygen lifetimes are increased in the membrane environment
independent of the mode of generation. It can diffuse across
membrane interfaces but the significance of this in biological
membranes is questionable.

Finally temperature can modulate photomodification rates,
probably through effects on sensitizer association with the membrane
and possibly by increased oxygen solubility above the phase
transition temperature of the membrane lipids.

       [Note: For completeness the reader should be aware
       that sensitization by psoralens has not been considered
       here. Psoralens act by non-singlet oxygen mechanisms
       on cellular DNA. Acridines and related sensitizers,
       which also affect DNA, have likewise not been treated.]

A number of questions concerning membrane photomodification remain unanswered. These include the following.

1.) What is (are) the membrane target(s) which are critical for cell killing (inactivation, lysis)?
2.) What is the most effective location for sensitizer in biological membranes?
3.) What are the triplet quantum yields for halogenated fluoresceins and the singlet oxygen quantum yields in membranes?
4.) What is the lifetime of singlet oxygen in biological membranes?
5.) What is the penetrability of singlet oxygen through biological membranes?
6.) Can sensitizer uptake account for the temperature dependence of membrane photomodification?

In conclusion membranes appear to be excellent targets for photomodification. Many sensitizers associate preferentially with the membrane, some generate singlet oxygen more efficiently there, oxygen solubility and hence oxygen concentrations are higher in the membrane interior, and singlet oxygen lifetimes are longer in the membrane interior. Since many of the molecular components of membranes are susceptible to photomodification reactions, conditions strongly favor membrane modification. Perhaps then it is understandable, as stated at the outset of this chapter, that membranes are so often identified as critical targets in cellular and organism photosensitization.

<u>Literature Cited</u>

1. Bellnier, D.A.; Dougherty, T.J. Photochem. Photobiol. 1982, 36, 43–47.
2. Kessel, D. Biochem. 1977, 16, 3443–3449.
3. Moan, J.; Pettersen, E.O.; Christensen, T. Br. J. Cancer 1979, 39, 398–407.
4. Kohn, K.; Kessel, D. Biochem. Pharmacol. 1979, 28, 2465–2470.
5. Volden, G.T.; Christensen, T.; Moan, J. Photobiochem. Photobiophys. 1981, 3, 105–111.
6. Henderson, B.W.; Bellnier, D.A.; Ziring, B.; Dougherty, T.J. Adv. Exper. Med. Biol. 1983, 160, 129–138.
7. Ehrenberg, B.; Malik, Z.; Nitzan, Y. Photochem. Photobiol. 1985, 41, 429–435.
8. Singer, S.J.; Nicolson, G.L. Science 1972, 175, 720–731.
9. Lux, S.; Shohet, S.B. Hospital Practice 1984, 19, 77–83.
10. Shohet, S.B.; Lux, S. Hospital Practice 1984, 19, 89–108.
11. Gilbert, D.L.; Ehrenstein, G. Cur. Top. Membr. Transp. 1985, 22, 407–421.
12. Lindig, B.A.; Rodgers, M.A.J. J. Phys. Chem. 1979, 83, 1683–1688.
13. Rodgers, M.A.J.; Snowden, P.T. J. Amer. Chem. Soc. 1982, 104, 5543–5545.
14. Parker, J.G.; Stanbro, W.D. Porphyrin Localization and Treatment of Tumors.; Alan R. Liss, Inc.: 1984; pp 259–284.
15. Pooler, J.P.; Valenzeno, D.P. Photochem. Photobiol. 1979, 30, 581–584.

16. Lindig, B.A.; Rodgers, M.A.J. Photochem. Photobiol. 1981, 33, 627-634.
17. Bezman, S.A.; Burtis, P.A.; Izod, T.P.J.; Thayer, M.A. Photochem. Photobiol. 1978, 28, 325.
18. Gorman, A.A.; Lovering, G.; Rodgers, M.A.J. Photochem. Photobiol. 1976, 23, 399-403.
19. Eisenberg, W.C.; Taylor, K.; Grossweiner, L.I. Photochem. Photobiol. 1984, 40, 55-58.
20. Sandberg, S.; Glette, J.; Hopen, G.; Solberg, C.O.; Romslo, I. Photochem. Photobiol. 1981, 34, 471-475.
21. Pooler, J.P.; Valenzeno, D.P. Photochem. Photobiol. 1979, 30, 491-498.
22. Valenzeno, D.P.; Pooler, J.P. Photochem. Photobiol. 1982, 35, 343-350.
23. Pooler, J.P.; Girotti, A.W. hotochem. Photobiol.1986, 44, 495-499.
24. Pooler, J.P. Photochem. Photobiol. 1986, 43, 263-266.
25. Cook, J.S.; Blum, H.F. J. Cell. Comp. Physiol. 1959, 53, 41-60.
26. Valenzeno, D.P. Photochem. Photobiol. 1984, 40, 681-688.
27. Valenzeno, D.P. I.E.E.E. J. Quant. Electronics 1984, QE20, 1439-1441.
28. Waggoner, A. J. Membr. Biol. 1976, 27, 317-334.
29. Easton, T.G.; Valinsky, J.E.; Reich, E. Cell 1978, 13, 475-486.
30. Valinsky, J.E.; Easton, T.G.; Reich E. Cell 1978, 487-499.
31. Schlegel, R.A.; Phelps, B.V.; Waggoner, A.; Terada, L.; Williamson, P. Cell 1980, 20, 321-328.
32. Kalyanaraman, B.; Sieber, F. Photochem. Photobiol. 1986, 43, 28s.
33. Lelkes, P.I.; Miller, I.R. J. Membr. Biol. 1980, 52, 1-15.
34. Bagchi, B.; Basu, S. Photochem. Photobiol. 1979, 29, 403-405.
35. Pooler, J.P.; Valenzeno, D.P. Photochem. Photobiol. 1978, 28, 219-228.
36. Varnadore, W.E.; Arrieta, R.T.; Duchek, J.R.; Huebner, J.S. J. Membr. Biol. 1982, 65, 147-153.
37. Floyd, R.A. Biochem. Biophys. Res. Commun. 1980, 96, 1305-1311.
38. Rodgers, M.A.J. Chem. Phys. Lett. 1981, 78, 509-514.
39. Rodgers, M.A.J. J. Phys. Chem. 1981, 85, 3372-3374.
40. Minch, M.J.; Shah, S.S. J. Org. Chem. 1979, 44, 3252-3255.
41. Wade, M.J.; Spikes, J.D. Photochem. Photobiol. 1971, 14, 221-224.
42. Emiliani, C.; Delmelle, M. Photochem. Photobiol. 1983, 37, 487-490.
43. Sandberg, S.; Romslo, I. Clin. Chim. Acta 1981, 109, 193-201.
44. Kessel, D.; Kohn, K.I. Cancer Res. 1980, 40, 303-307.
45. Suwa, K.; Kimura, T.; Schaap, A.P. Biochem. Biophys. Res. Commun. 1977, 75, 785-792.
46. Moan, J.; Sommer, S. Photochem. Photobiol. 1984, 40, 631-634.
47. Dougherty, T.J.; Boyle, D.G.; Weishaupt, K.R.; Henderson, B.A.; Potter, W.R.; Bellnier, D.A.; Wityk, K.E. Adv. Exptl. Med. Biol. 1983, 160, 3-13.
48. Pryor, W.A. Photochem. Photobiol. 1978, 28, 787-801. Deziel, M.R.; Girotti, A.W. J. Biol. Chem. 1980, 255, 8192-8198.

49. Rodgers, M.A.J. Photochem. Photobiol. 1983, 37, 99-103.
50. Girotti, A.W. J. Biol. Chem. 1978, 253, 7186-7193.
51. Deziel, M.R.; Girotti, A.W. Photochem. Photobiol. 1980, 31, 593-596.
52. Reyftman, J.P.; Santus, R.; Moliere, P.; Kohen, E. Photobiochem. Photobiophys. 1985, 9, 183-192.
53. Blum, A; Grossweiner, L.I. Photochem. Photobiol. 1985, 41, 27-32.
54. Murasecco, P.; Oliverso, E.; Braun, A.M.; Monnier, P. Photobiochem. Photobiophys. 1985, 9, 193-201.
55. Keene, J.P.; Kessel, D.; Land, E.J.; Redmond, R.W.; Truscott, T.G. Photochem. Photobiol. 1986, 43, 117-120.
56. Reddi, E.; Jori, G.; Rodgers, M.A.J.; Spikes, J.D. Photochem. Photobiol. 1983, 38, 639-645.
57. Gandin, E.; Lion, Y.; Van de Vorst, A. Photochem. Photobiol. 1983, 37, 271-278.
58. Fleming, G.R.; Knight, A.W.E.; Morris, J.M.; Morrison, R.J.S.; Robinson, G.W. J. Amer. Chem. Soc. 1977, 99, 4306-4311.
59. McClure, D.S.; Blake, N.W.; Hanst, P.L. J. Chem. Phys. 1954, 22, 255-258.
60. Bowers, P.G.; Porter, G. Proc. Roy Soc. (Lond.) 1967, 296A, 435-441.
61. Reddi, E.; Rodgers, M.A.J.; Spikes, J.D.; Jori, G. Photochem. Photobiol. 1984, 40, 415-421.
62. Lee, P.C.; Rodgers, M.A.J. J. Phys. Chem. 1983, 87, 4894-4898.
63. Miyoshi, N.; Tomita, G. Z. Naturforsch. 1979, 34b, 339-343.
64. Rodgers, M.A.J.; Lee, P.C. J. Phys. Chem. 1984, 88, 3480-3484.
65. Gorman, A.A.; Rodgers, M.A.J.; Chem. Phys. Lett. 1978, 55, 52-54.
66. Kraljic, I.; Barboy, N.; Leicknam, J.P. Photochem. Photobiol. 1979, 30, 631-633.
67. Matheson, I.B.C.; Lee, J.; King, A.D. Chem. Phys. Lett. 1978, 55, 49-51.
68. Miyoshi, N.; Tomita, G. Z. Naturforsch. 1978, 33b, 622-627.
69. Sconfienza, C.; Van de Vorst, A.; Jori, G. Photochem. Photobiol. 1980, 31, 351-357.
70. Blum, H.F.; Pace, N.; Garrett, R.L. J. Cell. Comp. Physiol. 1937, 9, 217-228.
71. Davson, H.; Ponder, E. J. Cell. Comp. Physiol. 1940, 67-74.
72. Blum, H.F.; Kauzmann, E.F. J. Gen. Physiol. 1954, 37, 301-311.
73. Ito, T. Photochem. Photobiol. 1981, 33, 117-120.
74. Braganza, L.F.; Blott, B.H.; Coe, T.J.; Melville, D. Biochim. Biophys. Acta 1983, 731, 137-144.
75. Suwa, K.; Kimura, T.; Schaap, A.P. Photochem. Photobiol. 1978, 28, 469-473.

Chapter 4

Identifying Singlet Oxygen in Chemical, Photochemical, and Enzymic Reactions

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Application of ultrasensitive Ge-based near IR
spectroscopy to studies of singlet oxygen in solution
at ambient temperature has produced new and unambiguous
results. From photosensitization comes evidence of
singlet oxygen generation by dyes in solvents,
including H_2O; quenching of singlet oxygen by vitamin
C; and of singlet oxygen-solvent interaction. The
tetracyclines show direct correlation between the
efficiency of singlet oxygen generation and their
clinical phototoxicity. Biological singlet oxygen, the
observation of enzyme systems generating singlet
oxygen, was found for the peroxidases —
myeloperoxidase, lactoperoxidase and chloroperoxidase.
Lipoxygenase exhibits a weak singlet oxygen
luminescence. Spectral evidence of singlet oxygen
generation in the thermal dissociation of the
polycyclic endoperoxides is now available. A highly
efficient low-temperature source of singlet oxygen was
discovered in the reaction of triethylsilane with
ozone.

In current chemical, photochemical and biological research, singlet
oxygen is often proposed as the reactive intermediate (1-2). The
transient presence of singlet oxygen is generally deduced from
chemical products, scavenger trapping and other secondary evidence.
Many of these secondary effects can equally indicate the presence
of other reactive intermediates — $O_2^{\overline{\cdot}}$, OH·, HOO· — and also can not
distinguish between sigma and delta singlet oxygen. An
unambiguous identification of singlet ($^1\Delta_g$) oxygen molecules in
solution is crucial to the growth of this research field. Over the
last number of years we have developed ultrasensitive
spectrophotometers for the near infrared, initially based on a
thermoelectrically cooled lead sulfide detector, optimized optics,
integrators and data processors (3), later more sensitive
instruments based on a germanium detector (4). The present
spectrometer covers the range of 1.0 to 1.7 micron, and is capable
of detecting both the (0,0) and (0,1) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transitions of the
oxygen molecule at 1268 nm and 1586 nm, respectively.

In 1976 Krasnovsky (5) and in 1978 Byteva and Gurinovitch (6) were able to observe the photosensitized 1270 nm emission of singlet oxygen in CCl_4 solution. In CCl_4 singlet oxygen has one of the longest observed solution lifetimes. We have used the high-sensitivity luminescence spectrometer to compare singlet oxygen emission characteristics in different solvents, to follow the kinetics of reactions of singlet oxygen, to discriminate between mechanistic alternatives, and to discover new chemical and biological sources of singlet oxygen. Although visible emission spectroscopy played a critical role in the discovery of the chemical generation (7) and the subsequent characterization of singlet oxygen in the red chemiluminescence of hydrogen peroxide-hypochlorite reaction (8-9), this paper is only concerned with infrared emission. This presentation has the following outline: (i) a brief description of the latest version of the luminescence spectrometer; (ii) electronic energy transfer generation of singlet oxygen in a) spectroscopy of dissolved oxygen, b) photosensitization by dyes of biological interest, c) kinetics of singlet oxygen reaction in aqueous solution, d) photosenitization by drugs; (iii) enzymic generation of singlet oxygen in a) microbicidal enzymes - myeloperoxidase and lactoperoxidase, b) plant enzymes - chloroperoxidase c) biosynthetic enzymes - lipoxygenase; (iv) thermal generation; and (v) a new source of chemical generation.

Instrumentation

In Figure 1 is shown the high sensitivity luminescence spectrometer. The spectrometer consists of a Spex Minimate II, f/4.0 monochromator (Spex Industries, Metuchen, N.J.), fitted with a 1.25 micron blazed grating, liquid nitrogen cooled germanium detector 403L (Applied Detector, Fresno, CA), followed by a low noise amplifier PAR model 113 (E.G.&G. Princeton Applied Research, Princeton, N.J.), lock-in amplifier (PAR model 5207), leading to a Spex Datamate with digital storage and printout. An optical filter, (F), Corning CS 7-56 is placed before the entrance slit of the monochromator to reject second order interfering emissions. A collecting lens, (L), focuses the monochromator output onto the detector crystal.

The estimated sensitivity of the luminescence spectrometer is 10^8 photons per second at 1270 nm. The estimate is based on the assumption that the thermal dissociation of 1,4-dimethyl naphthalene-1,4-endoperoxide leads to a 100% yield of O_2 $(^1\Delta_g)$ (10-11) in carbon tetrachloride solution at 50°C. The assumed lifetime in carbon tetrachloride is 20 msec at this temperature (12).

Electronic Energy Transfer Generation of Singlet Oxygen

The first indication that a light-dependent activation might exist for molecular oxygen was the discovery of the spontaneous oxidation of naphthacene in the presence of light and air by Fritzsche in 1867 (13), followed by the discovery by Raab in 1900 (14) of damage to living tissue by the synergistic effect of light, air and organic dye molecules. Both these effects are now recognized as examples of photooxidation reactions. Much interest centered on these reactions in the early part of this century, resulting in their characterization by product isolation and identification and kinetic analysis. Kautsky and de Bruijn in 1931 (15) speculated
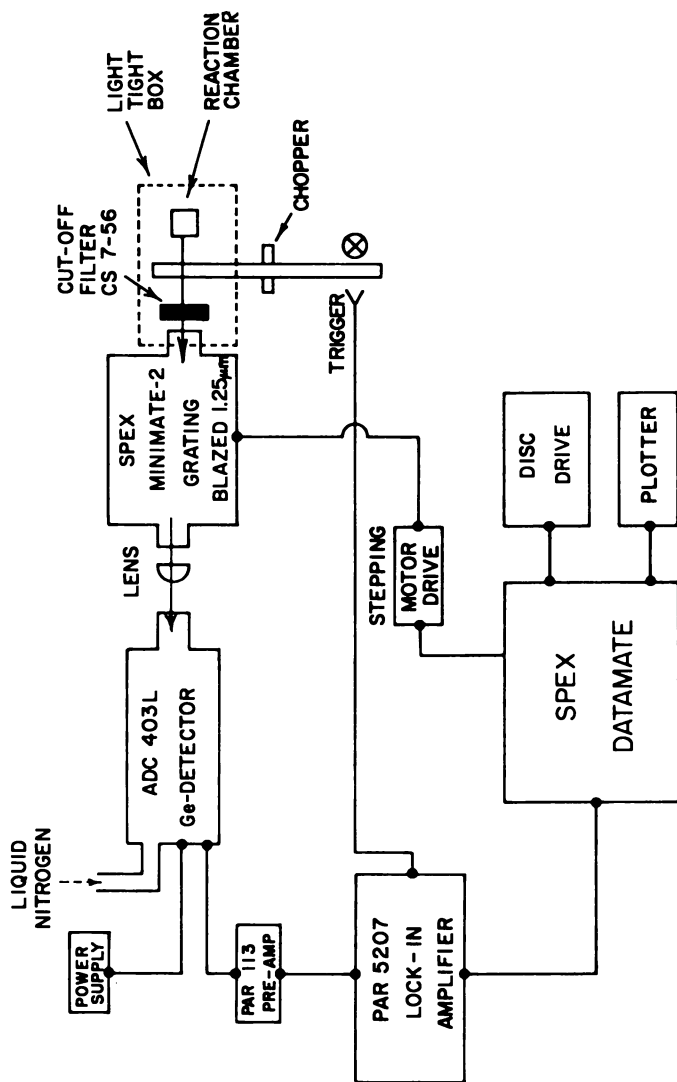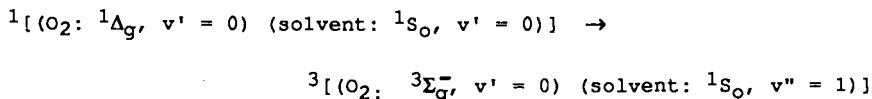
Figure 1. High-sensitivity one micron region luminescence spectrometer.

that photooxidation mechanisms involved singlet oxygen. However, by the late 1930's Kautsky's speculations were buried under severe criticism by his contemporaries (16-18). The recognition of singlet oxygen as a chemical species came with the recognition of the chemical generation of singlet oxygen in the simple chemical reaction of hypochlorite ion with hydrogen peroxide in the spectroscopic discovery in 1963 by Khan and Kasha (7). Reexamination of singlet oxygen as a reactive intermediate in photooxidation reactions ensued (19-21).

Direct absorption of light to generate singlet oxygen is highly improbable because of the low oscillator strength of transition between the ground triplet state of the oxygen molecule and its first two excited singlet states (22-23). Kawaoka, Khan and Kearns (24-25) in 1967 established the theoretical basis of photosensitized generation of singlet oxygen in the quenching of organic triplet states. This electronic energy transfer process circumvents the restriction of direct optical excitation and is the highly efficient process which forms the key step in the photooxidation reactions. Direct spectroscopic verification of the energy transfer generation of singlet oxygen in the gas phase followed (26-28). The first direct spectroscopic observation of sensitized generation of singlet oxygen in solution, detected by the 1268 nm near infrared emission, is by Krasnovsky (5). He used photomultiplier detection, CCl_4 as solvent, and chlorophyll and related dyes as sensitizers. Khan and Kasha (3) developed an ultrasensitive near infrared spectroscopy and applied it to study singlet ($^1\Delta_g$) oxygen in solution.

Spectroscopy of Dissolved Oxygen Molecules: O_2 ($^1\Delta_g$)...Solvent Interaction. The electronic states of molecular oxygen in solution are the focus of this presentation. Spectroscopic investigation of molecular oxygen in solution indicates very little, if any, frequency shift from the gas phase luminescence frequency of singlet ($^1\Delta_g$) state of O_2 (3-4,29). However, using the Ge-based spectrometer, Chou and Khan (30) observed distinct new emission bands from oxygen saturated CCl_4, $CDCl_3$, $C_2F_3Cl_3$ and $C_{10}F_{18}$. These new bands are much weaker (ratio ~ 1/300 to ~ 1/550) and red shifted from the (0,0) vibronic band of $^1\Delta_g \rightarrow {}^3\Sigma_g^-$. The appearance of these bands is consistent with a simultaneous electronic-vibrational transition involving the $^1\Delta_g$ state of oxygen and a vibrational state of the solvent molecule:

$$^1[(O_2: {}^1\Delta_g, v' = 0) \text{ (solvent: } {}^1S_o, v' = 0)] \rightarrow$$

$$^3[(O_2: {}^3\Sigma_g^-, v' = 0) \text{ (solvent: } {}^1S_o, v'' = 1)]$$

The spectrum in Figure 2 shows clearly the interaction of dissolved singlet oxygen with solvent molecules.

Photosensitized Singlet Oxygen Emission by Dyes of Biological Interest in Liquid Solutions. Methylene blue sensitized generation of singlet oxygen in aqueous solution is a commonly used system for studying the photochemical (31) and photobiological (32) effects of the oxygen molecule. From secondary evidence it was believed that
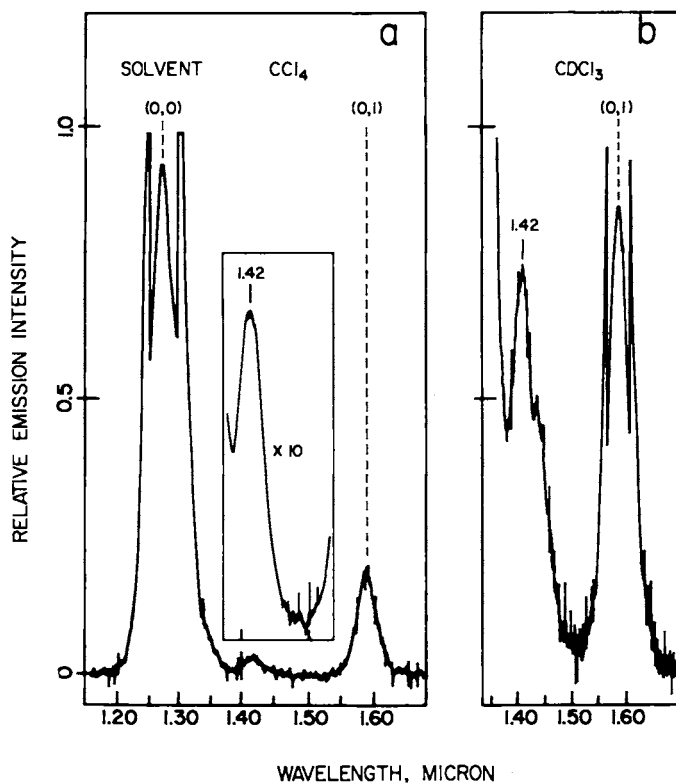
Figure 2. Photosensitized emission of dissolved molecular oxygen
at room temperature, broad band excitation, 320–485 nm. (a) Solvent
CCl_4, sensitizer benzophenone, oxygen gas saturated. Spectrum
displays the normal (0,0) and (0,1) emissions at 1.28 micrometer
and 1.58 micrometer, respectively. New emission band appears at
1.42 micrometer. The insert displays the new band at ten times
expansion. (b) Solvent $CDCl_3$, sensitizer perfluorobenzophenone,
oxygen saturated. New emission band at 1.42 micrometer. (Adapted
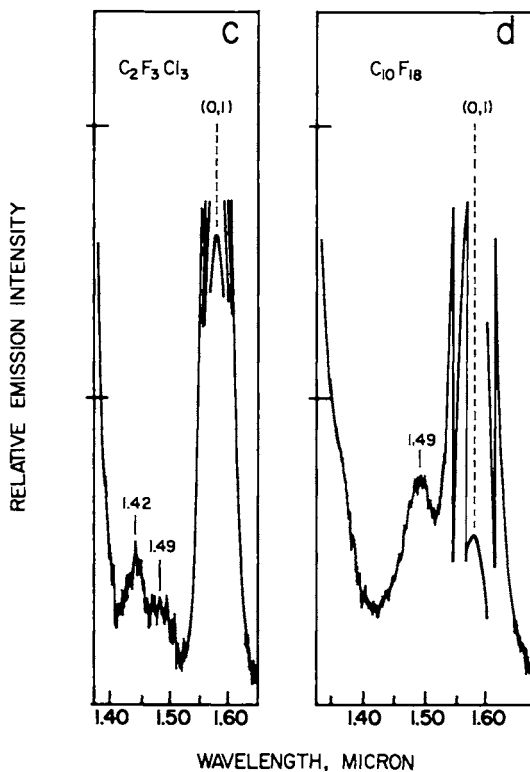from Reference 30.)

Figure 2.--Continued. (c) Solvent $C_2F_3Cl_3$, sensitizer perfluoro-
benzophenone, oxygen saturated. Spectrum shows two new emission
bands at 1.42 micrometer and 1.49 micrometer. (d) Solvent $C_{10}F_{18}$,
sensitizer perfluorobenzophenone, oxygen saturated.
Spectrum shows the new emission band at 1.49 micrometer. (Adapted
from Reference 30.)

singlet oxygen is efficiently generated in this system, but,
because of its extremely short lifetime in water ($\underline{33}$), direct
singlet oxygen emission was not observable. The inability to
observe singlet oxygen emission in aqueous media raised questions
in evaluating this research. Using our spectrometer based on the
PbS detector, we observed the emission shown in Figure 3a. The
1268 nm emission of singlet oxygen is superimposed on the tail of
the methylene blue emission. This is the first spectral evidence
of singlet oxygen generation in aqueous solution. Note that in the
chemiluminescence of hydrogen peroxide-hypochlorite reaction in
aqueous solution, the emission originates in the gas phase inside
reaction bubbles ($\underline{34}$).

Hematoporphyrin sensitized generation of singlet oxygen (Figure 3b)
is an especially interesting example because this photodynamic
pigment is now used with success in the phototherapy of cancer via
an apparent singlet oxygen mechanism ($\underline{35}$). Injected into the
bloodstream, the pigment preferentially adsorbs on the tumor and is
photoexcited using fiber optics connected to a dye laser source.
Krasnovsky ($\underline{36}$) has reported an extensive study of singlet oxygen
emission in CCl_4 using various other porphyrins as
photosensitizers.

3,4-benzpyrene, an atmospheric pollutant and a notorious
carcinogenic agent ($\underline{37-38}$), is also a photosensitizer of singlet
oxygen, as shown in Figure 3c.

Kinetics of Singlet Oxygen Reaction in Aqueous Solution: Vitamin C
Quenching of Singlet Oxygen.($\underline{39}$) L-ascorbic acid, an aqueous phase
antioxidant in both plant and animal physiology is present in all
eucaryotic organisms ($\underline{40-42}$) and is a topic of lively interest both
in chemistry and medicine in recent times ($\underline{43}$). Chou and Khan
synthesized a water-soluble photosensitzer, chrysene sodium
sulfonate, to photosensitize singlet oxygen in aqueous solution.
The quenching of singlet oxygen by vitamin C was studied by
directly monitoring the 1268 nm emission. On comparing quenching
of photogenerated singlet oxygen in H_2O and D_2O solutions, a marked
isotope effect was seen. Stern-Volmer constants are $K^Q_{H2O} = 8.30$ x
10^6 and $K^Q_{D2O} = 2.50$ x 10^6 $M^{-1}s^{-1}$. The isotope effect points to a
chemical quenching of singlet oxygen by vitamin C, possibly by H-
atom abstraction. Figures 4 & 5 summarize the results. Note that
the chrysene sodium sulfonate sensitized singlet oxygen emission
spectrum in aqueous medium does not have any overlapping emission
from the sensitizer, compare with the methylene blue spectrum,
Figure 3a.

Photosensitization by Drugs: Phototoxicity of the Tetracyclines.
Tetracyclines are one of the most frequently prescribed group of
antibiotics, deriving their bacteriostatic effect by preventing the
binding of aminoacyl-tRNA to the aminoacyl (A) site of the ribosome
($\underline{44}$). A well known side effect of tetracycline therapy is
cutaneous phototoxicity. Clinical estimates of phototoxicity in a
series of tetracyclines clearly indicates that chloro-derivatives
(chlorotetracycline and demeclocycline) are the most phototoxic,
tetracycline itself being less so, and minocycline having no
associated phototoxicity ($\underline{45-49}$). In vitro chemical studies of
tetracycline photosensitization have suggested that singlet oxygen
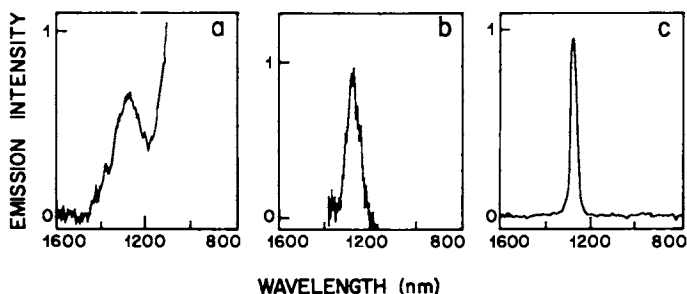is the reactive intermediate being generated ($\underline{50-53}$). Recently

Figure 3.   Dye photosensitized (0,0) $^1\Delta_g \to {}^3\Sigma_g^-$ emission of
oxygen at 1268 nm in liquid solution at room
temperature, excitation 320- 485 nm.   (a)
Sensitizer, methylene blue, solvent, H_2O, O_2
saturated. (b) Sensitizer hematoporphyrin, solvent
CCl_4, O_2 saturated.   (c) Sensitizer 3,4-benzpyrene,
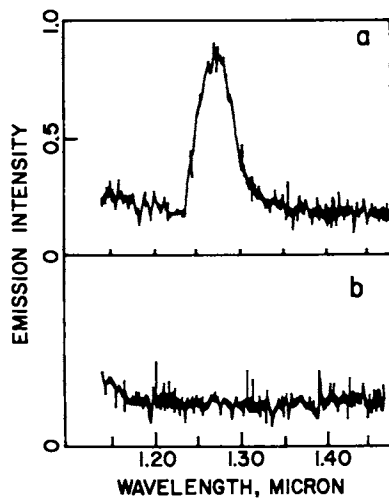solvent CCl_4, O_2 saturated. (Adapted from Ref. 3).

Figure 4.   (a) (0,0) $^1\Delta_g \to {}^3\Sigma_g^-$ emission of dissolved molecular
oxygen in [^{1}H]H_2O, sensitized by sodium chrysene
sulfonate (10^{-3} M), excitation 320-485 nm, at room
temperature.   (b) Total quenching of the 1.28
micron emission on addition of L-ascorbic acid
(0.20 M). (Taken from Ref. 39).

Hasan and Khan (54) directly monitored the photosensitized
generation of singlet oxygen in the tetracycline series,
demeclocycline, tetracycline and minocycline. They found one-to-
one correspondence between the efficiency of the singlet oxygen
generation and the phototoxicity of the antibiotic. The tentative
conclusion is that singlet oxygen is the reactive intermediate in
the phototoxicity of tetracyclines. Figure 6 presents these
findings.

Enzymatic Generation of Singlet Oxygen

After the chemical discovery of singlet oxygen, many attempts were
made to implicate singlet oxygen in biological and biochemical
reactions. The main tools used were monitor of ultraweak visible
luminescence, chemical product and chemical scavenger techniques,
and deuterium kinetic effects. Although these techniques are non-
specific in detecting singlet oxygen, a number of valuable
suggestions emerged. Krishnamurty and Simpson (55) were the first
to suggest the possible involvment of singlet oxygen in enzymatic
reactions, using the fungus, Aspergillus flavus. Aspergillus
flavus produces the enzyme quercitinase that oxidizes quercetin to
give a depside clevage product. Matsuura, et al, (56) had earlier
obtained the same depside following photosensitized oxidation,
presumably a singlet oxygen mediated reaction. Krishnamurty and
Simpson concluded that singlet oxygen was the reactive species
enzymatically generated by quercitinase. Another important
suggestion, due to Allen et al and based on the observation of
ultraweak visible chemiluminescence, suggested that singlet oxygen
might be a product of the metabolism of phagocytosing
polymorphonuclear leukocytes (57). These suggestions resulted in
an extensive search for singlet oxygen in enzymic and biological
processes but no clear evidence of singlet oxygen generation
emerged either in biology or in biochemistry.

Using the Ge based spectrometer, Khan, Gebauer and Hager (58)
published the first spectrum of singlet oxygen emission from an
enzymic reaction, the chloroperoxidase/H_2O_2/Cl^- system, providing
incontrovertible evidence of singlet oxygen generation in an
enzymic system. Kanofsky (59) has also studied enzymic generation
of singlet oxygen in this system and others with a kinetic
apparatus based on a Ge detector by monitoring 1270 nm emission
using interference filters.

Microbicidal Enzymes: Myeloperoxidase: Polymorphonuclear
Leucocytes. Probably the most significant enzyme of
polymorphonuclear leucocytes (PMN) involved in the physiological
defense against foreign bodies is myeloperoxidase (MPO). MPO was
originally isolated by Agner and is estimated to constitute greater
than 5% of the dry weight of the human PMN (60). Mainly through
the pioneering work of Klebanoff (61), the potent antimicrobial
system of MPO/H_2O_2/halide was characterized. The MPO/H_2O_2/halide
antimicrobal system is toxic to a wide variety of organisms:
bacteria (61-62), fungi (63), viruses (64), mycoplasma (65),
chlymadia (66), protoza (67) and multicellular organisms such as
schistosmula of Schistosoma mansoni (68). The peroxidase is also
toxic to certain mamallian cells, e.g. spermatozoa (69),
erythrocytes (70), leucocytes (71), platelets (72) and tumor cells
(73), and inactivates certain soluble mediators such as the
chemotactic factor, C5a (74). The peroxidase can also transform
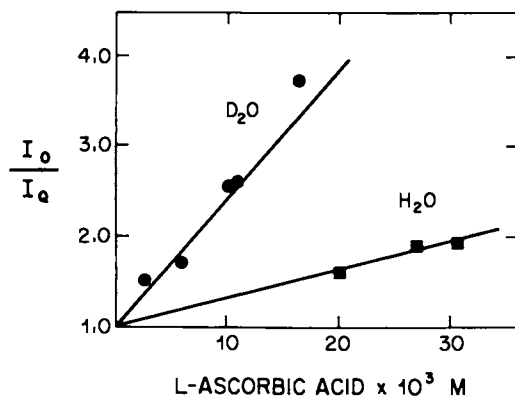
Figure 5:   Stern-Volmer plot of 1.28 micron emission of
            singlet delta molecular oxygen as a function of
            ascorbic acid concentration. (Taken from Ref. 39).
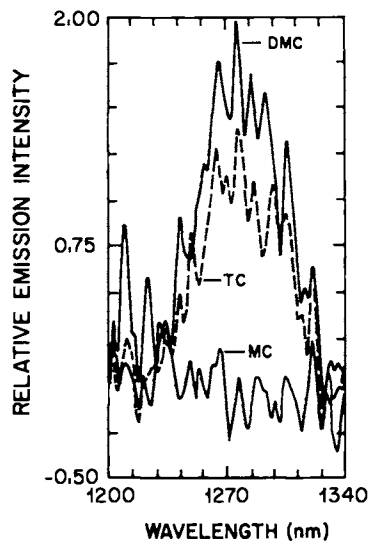

Figure 6:   Near IR singlet oxygen emission photosensitized by
            demeclocycline (DMC), tetracycline (TC),
            minocycline (MC); oxygen saturated solvent [99.4%
            CCl_4/0.6 % Me_2SO (vol/vol)] at room temperature.
            (Taken from Ref. 54).

prostaglandins (75), thus possibly plays a regulatory role in immune function by modulating the inflammatory response.

The mechanism of action of the MPO system is complex and the currently accepted view is as follows (76):

$$H_2O_2 + Cl^- + H^+ \;\rightarrow\; H_2O + HOCl \;\leftrightarrow\; H^+ + OCl^-$$
$$\text{MPO}$$
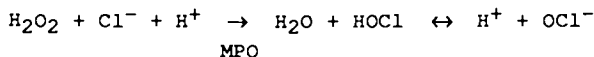
Recognizing the classic singlet oxygen generating reaction of hydrogen peroxide-hypochlorite (8):

$$H_2O_2 + OCl^- \rightarrow O_2(^1\Delta_g) + H_2O + Cl^-$$
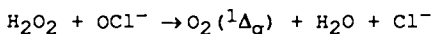
in the MPO enzyme mechanism, Rosen and Klebanoff decided to explore the singlet oxygen generating potential of the enzyme system using chemical scavengers and deuterium kinetic effects. They concluded that chemical evidence supports singlet oxygen generation in the MPO system (77).

Using our Ge-spectrometer and with the gift of a sample of myeloperoxidase from Drs. Rosen and Klebanoff, we have obtained the critical spectral evidence of singlet oxygen generation from the MPO/H_2O_2/Br^- system (Figure 7). We were also able to estimate the efficiency of singlet oxygen generation in this system to be about 0.5, i.e., two H_2O_2 molecules yield one molecule of $O_2(^1\Delta_g)$ in this enzymatic system (78). Kanofsky et. al. (79) have also seen singlet oxygen generation in the MPO system. They emphasize the non-physiological conditions of the experiments.

Lactoperoxidase: Milk and Saliva. Lactoperoxidase (LPO) is secreted into saliva by the human salivary glands and is also produced by the mammary glands and found in high concentration in milk, particularly bovine milk. Theorell, et al. (80-81) were the first to obtain a highly purified prepartion of LPO enzyme crystals. Klebanoff established the antimicrobial activity of the LPO/H_2O_2/halide system (61). We have obtained singlet oxygen emission from the LPO/H_2O_2/Br^- reaction with the Ge spectophotometer. Kanofsky (82) has performed a kinetic study of the LPO reaction. Our estimated efficiency of singlet oxygen generation from the LPO reaction is comparable to the efficiency of the MPO reaction, bearing out their similar antimicrobial action (83). MPO, however, occurs inside the granules embedded in the membrane of the PMN and is released into the phagosome on degranulation by the activated PMN, in contrast to LPO which is not confined in vacules.

Plant Enzymes: Chloroperoxidase. Chloroperoxidase (CPO), was originally isolated and characterized by Morris and Hager (84). CPO has an effective catalase-like activity, as well as exhibiting the classical peroxidative and halogenating activity of a peroxidase (85). The enzyme can utilize both chloride and bromide ions for enzymic halogenation. Khan, Gebauer, and Hager examined the CPO/H_2O_2/Cl^- enzyme system for singlet oxygen generation and

obtained a strong 1268 nm emission, shown in Figure 8 (58). This
is the first reported spectrum of singlet oxygen generated in an
enzymic system.

<u>Biosynthetic Enzymes</u>: <u>Lipoxygenase</u> (86). The lipoxygenase
mediated oxygen molecule reaction with polyunsaturated fatty acids
yielding hydroperoxides is of fundamental importance in lipid
biochemistry and is the initial step in the biosynthesis of a host
of biologically and medically important molecules, the
prostaglandins and leukotrienes (87-88). We observed a weak
singlet oxygen emission from the lipoxygenase reaction with either
linolenic acid or with arachidonic acid as substrate. Using the
Ge-spectrometer, we searched for 1268 nm emission of singlet oxygen
from Lipoxidase, Type 1/Na-linoleate/O_2 and Lipoxidase, Type 1/Na-
arachidonate/O_2 reactions at room temperature. A typical
experiment consists of [Lipoxidase, Type 1 (Sigma Chemical Co.),
100 μg/ml; Na-linoleate (Sigma Chemical Co.), 40 mM; 0.1 M tris-
HCl, pH 9.2 buffer with slow continuous bubbling of O_2, total
volume 15 ml], [20 scans, between 1240-1300 nm, 1nm increment, 3
sec per nm, background subtraction]. Recently Kanofsky and Axelrod
(89), using a Ge-kinetic spectrometer, observed the 1268 nm singlet
oxygen emission from the oxidation of linoleic acid catalyzed by
soybean lipoxygenase isozymes, mainly from lipoxygenase-3. From
their investigation under optimal singlet oxygen generating
conditions, they concluded that a Russell like mechanism (90) of
peroxy radical recombination leading to singlet oxygen generation
was quite plausible.

<u>Thermal Generation</u>: <u>Dissociating Endoperoxide</u>

Singlet oxygen, O_2 ($^1\Delta_g$), reacts with polycyclic hydrocarbons to
produce endoperoxides which, upon heating, regenerate molecular
oxygen and the parent hydrocarbon (10,91-93). In the case of some
of the transannular peroxides of the napthalene and anthracene
series, chemical reactivity studies have shown that a large
fraction, if not all, of the regenerated oxygen appears to be in
the singlet excited state (11,94-95). Wilson, Khan, and Mehrotra
(96) chose two endoperoxides, 1.4-dimethyl-napthalene-1,4-
endoperoxide and 1,4-dimethoxy-9,10-diphenyl-anthraene-1,4-
endoperoxide to spectrally investigate the generation of singlet
oxygen in the thermal dissociation of these endoperoxides. See
Figure 9. Also shown in the figure is the observed spectral
distribution of the thermal emission of the solvent at the same
temperature. Note that the thermal spectral maximum displayed is
an apparent one, not a true maximum. Chou and Frei (97) have
reported the 1270 nm emission of singlet oxygen from the thermal
dissociation of 1,4-dimethyl napthalene at room temperature.

<u>Chemical Generation</u>: <u>Triethylsilane-Ozone Reaction</u>

Corey, Mehrotra and Khan (98) recently examined the reaction of
trialkylsilane with ozone at -75°C in inert organic solvents and
found a highly efficient low-temperature source for singlet delta
oxygen. Using the Ge-spectrometer, they characterized a freely
diffusing singlet delta oxygen molecule generated from a reaction
intermediate. The intermediate is trialkyl silyltrioxide
[$(C_2H_5)_3SiOOOH$] with an approximate half life of 150 seconds in
methylene chloride at <u>ca</u> -60°C. Chemical trapping experiments

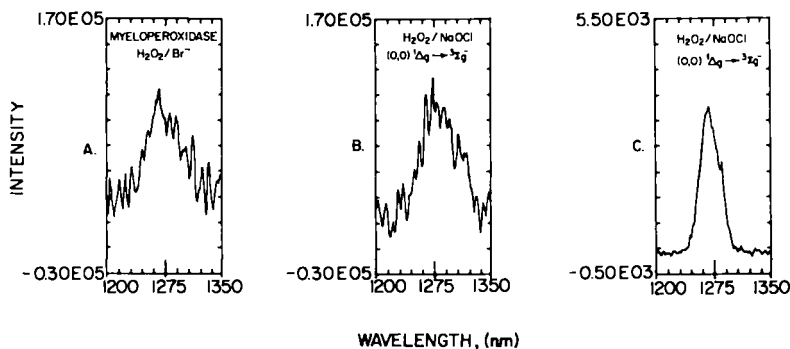WAVELENGTH, (nm)

Figure 7:   Chemiluminescence emission spectra at room
             temperature. Spectra displayed from 1200-1350 nm,
             intensity scale is proportional to the number of
             photons emitted by the source (negative numbers on
             the scale are due to instrumental background
             subtraction). A. The 1268 nm emission of singlet
             delta dioxygen from myeloperoxidase reacting with
             H_2O_2 in the presence of Br^-. B. The 1268 nm
             emission of singlet delta dioxygen from the
             standard singlet oxygen generating reaction OCl^-
             •H_2O_2 under comparable conditions. C. The 1268 nm
             emission from the OCl^-•H_2O_2 under near optimum
             detection conditions. (Adapted from Ref. 78).
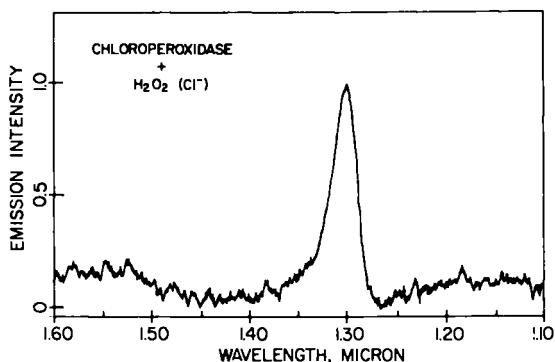

Figure 8:   Near-infrared singlet oxygen chemiluminescence
             spectrum from the enzymatic reaction of
             chloroperoxidase with H_2O_2 in the presence of Cl^-.
             (Adapted from Ref. 58).
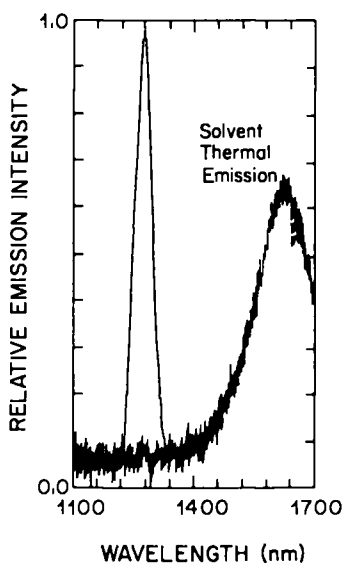
Figure 9:   1,4-dimethoxy-9,10-diphenylanthracene-1,4-
            endoperoxide in CCl_4 at 50°C. Also shown are the
            solvent thermal emission giving an apparent maximum
            at ca 1600 nm due to a drop in detector
            sensitivity. Two scans are shown, one taken
            immediately after addition of the endoperoxide, the
            other after its complete decomposition. (Adapted
            from Ref. 96).

estimated the efficiency of singlet oxygen generation to be 91%.
The reaction scheme is as follows:

$$(C_2H_5)_3SiH + O_3 \rightarrow (C_2H_5)_3SiOOOH \rightarrow (C_2H_5)_3SiOH + O_2 \ (^1\Delta_g)$$
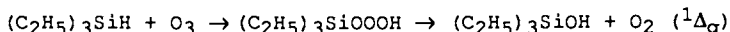
This convenient, highly efficient, low-temperature singlet oxygen
source may have wide application in the synthesis of thermally
labile singlet oxygen reaction products of hydroperoxides and
endoperoxides.

Conclusion. Singlet oxygen research represents a new cross-
disciplinary effort. The methodologies of chemical kinetics,
photochemistry, and photobiology can all be applied to the problem,
subject to a single restraint, that the presence of singlet oxygen
is unambiguously established. Spectroscopic techniques are the
natural choice to fulfill this task. In spectroscopy, however,
detecting this low oscillator strength transition in a complex
matrix is difficult. Near infrared detectors are not very
sensitive. The emission of the $^1\Delta_g$ electronic state in solution is
one of the weakest known, since high metastability makes this state
extremely susceptible to solvent quenching. The detection limit in
CCl_4 with our instrument is about 10^{-10} moles/sec. In H_2O singlet
oxygen is more drastically quenched and only one photon is emitted
for every 10^9 singlet oxygen molecules generated, putting the
detection limit in aqueous media at 10^{-6} moles/sec.

Literature Cited

1. Singlet Molecular Oxygen, Benchmark Papers in Organic
    Chemistry; Schaap, A. P., Ed.; Dowden, Hutchinson and Ross:
    Stroudsburg, PA, 1976; Vol. 5.
2. Singlet O$_2$; Frimer, A. A., Ed.; CRC Press, Inc.: Boca Raton,
    FL, 1985; Vol. 1-4.
3. Khan, A. U.; Kasha, M. Proc. Natl. Acad. Sci. USA 1979, 76,
    6047-49.
4. Khan, A. U. J. Am. Chem. Soc. 1981, 103, 6516-17.
5. Krasnovsky, A. A., Jr. Biofisika 1976, 21, 748-49.
6. Byteva, I. M.; Guvinovitch, G. P. ZPrikl. Spektr. 1978, 29,
    154.
7. Khan, A. U.; Kasha, M. J. Chem. Phys. 1963, 39, 2105-6.
8. Khan, A. U.; Kasha, M. J. Am. Chem. Soc. 1970, 92, 3293-300.
9. Arnold, S. J.; Ogryzlo, E. A.; Witzke, H. J. chem. Phys. 1964,
    40, 1769-70.
10. Wasserman, H. H. ; Larsen, D. L. JCS Chem. Comm. 1972, 253-54.
11. Turro, N. J.; Chow M. -F.; Rigaudy, J. J. Am. Chem. Soc. 1981,
    103, 7218-24.
12. Krasnovsky, A. A., Jr. Chem. Phys. Lett. 1981, 81, 443-45.
13. Fritzsche, M. C. R. Acad. Sci. (Paris) 1867, 64, 1035-37.
14. Raab, O. Z. Biol. 1900, 39, 524-46.
15. Kautsky, H.; de Bruijn, H. Naturwissenschaften 1931, 19, 1043.

16. Gaffron, H. Biochem. Z. 1936, 287, 130-39.
17. Schenck, G. O. Naturwissenschaften 1948, 35, 28-29.
18. Khan, A. U. In Singlet O₂; Frimer, A. A., Ed.; CRC Press, Inc.: Boca Raton, FL, 1985; Vol. 1, Chapter 3.
19. Foote, C. S.; Wexler, S. J. Am. Chem. Soc. 1964, 86, 3879-80.
20. Corey, E. J.; Taylor, W. C. J. Am. Chem. Soc. 1964, 86, 3881-82.
21. McKeown, E.; Waters, W. A. J. Chem. Soc. (B) 1966, 1040-46.
22. Childs, W. H. J.; Mecke, R. Z.Phys. 1931, 68, 344-61.
23. Badger, R. M.: Wright, A. C.; Whitlock, R. F. J. Chem. Phys. 1965, 43, 4345-50.
24. Kawaoka, K.; Khan, A. U.: Kearns, D. R. J. Chem. Phys. 1967, 46, 1842-53.
25. Kawaoka, K.; Khan, A. U.: Kearns, D. R. J. Chem. Phys. 1967, 47, 1883-84.
26. Snelling, D. R. Chem. Phys. Lett. 1968, 2, 346-48.
27. Kearns, D. R.; Khan, A. U.; Duncan, C. K.; Maki, A. H. J.Am.Chem.Soc. 1969, 91 1039-40.
28. Wasserman, E.; Kuck, V. J.; Delavan, W. M.; Yeager, W. A. J.Am.Chem.Soc. 1969, 91, 1040-41.
29. Khan, A. U. Chem.Phys.Lett. 1980, 72, 112-14.
30. Chou, P.; Khan, A. U. Chem.Phys.Lett. 1984, 103, 281-84.
31. Gollnick, K. Adv.Photochem. 1968, 6, 1-122.
32. Spikes, J. D.; Livingston, R. Adv.Radiation Biol. 1969, 3, 29-121.
33. Monroe, B. In Singlet O₂; Frimer, A. A. ED.; CRC Press Inc.: Boca Raton, FL, 1985; Vol.1 Chapter 5.
34. Khan, A. U.; Kasha, M. Nature 1964, 204, 241-43.
35. Dougherty, T. J.; Kaufman, J. E.; Goldfarb, A,; Weishaupt, K. R.; Boyle,D.; Mittleman, A. Cancer Res. 1978, 38, 2628-35.
36. Krasnovsky, A. A. Jr. Photochem. Photobiol. 1979, 29, 29-36.
37. Selkirk, J. K. In Modifiers of Chemical Carcinogenesis: An Approach to the Biochemical Mechanism and Cancer Prevention; Slaga, T. J. ED.; Raven Press: New York. 1980; Chapter 1.
38. Khan, A. U.; Kasha, M. Ann. N. Y. Acad. Sci. 1970, 171, 24-32.
39. Chou, P.; Khan, A. U. Biochem.Biophys.Res.Commun. 1983, 115, 932-37.
40. Szent-Györgyi, A. Studies on Biological Oxidation, Barth: Leipzig, Germany, 1937.
41. Lind Bicentenary Symposium; Stewart, C. P. ED.; Edinburgh, Scotland, 1953.
42. Vitamin C; Burns, J. J. ED.; Ann. N. Y. Acad. Sci. 1961, Vol.92, Art.1.
43. Pauling, L. How to Live Longer and Feel Better; W.H.Freeman, New York, 1986.
44. Gale, E. F.; Cundliffe, E.; Reynolds, P.E.; Richmond, M. H.; Waring M. J. The Molecular Basis of Antibiotic Action; Wiley, New York, 1981; pp 448-53.
45. Cullen, S. I.; Catalano, P. M.; Helfman, R. J. Arch. Dermatol. 1966, 93, 77.
46. Schorr, W. F.; Monash, S. Arch Dermatol. 1963, 88, 134-38.
47. Frost, P.; Weinstein, G. D.; Gomez, E.C. JAMA 1971, 216, 326-29.
48. Zuehlke, R. L. Arch Dermatol. 1973, 108, 837-38.
49. Frank, S. B.; Cohen, J. H.; Minkim, W. Arch. Dermatol 1971, 103 520-21.
50. Blank, H.; Cullen, S. I.; Catalano, P. M. Arch. Dermatol. 1968, 97, 1-2.
51. Weibe, J. A. : Moore, D. E. J. Pharm. Sci. 1977, 66 186-89.

52. Hasan, T.; Kochevar, I. E.; McAuliffe, D. J.; Cooperman, B. S.; Abdulah, D. J. Invest. Dermatol. 1984, 83, 179-83.
53. Sandberg, S. O.; Glette, J.; Hopen, G.; Solberg, C. O. Photochem. Photobiol. 1984, 39, 43-48.
54. Hasan, T.; Khan, A. U. Proc. Natl. Acad. Sci USA 1986, 83, 4604-06.
55. Krishnamurty, H. G.; Simpson,F. J. J. Biol. Chem. 1970, 245, 1467-71.
56. Matsuura, T.; Matsushima, H.; Sakamoto, H. J. Am. Chem. Soc., 1967 89, 6370-71.
57. Allen, R. C.; Stjernholm, R. L.; Steele, R. H. Biochem. Biophys. Res. Commun. 1972, 47, 679-84.
58. Khan, A.U.; Gebauer, P.; Hager, L. P. Proc. Natl. Acad. Sci. USA 1983, 80, 5195-97.
59. Kanofsky, J. R. J. Biol. Chem. 1984, 259, 5996-00.
60. Agner, K. In Structure and Function of Oxidation-Reduction Enzymes; Akeson, A.; Ehrenberg, A., Eds.; Pergamon, Oxford, 1972; pp 329-35.
61. Klebanoff, S. J. J. Exp. Med. 1967, 126, 1063-78.
62. Klebanoff, S. J. J. Bacteriol. 1968, 95 2131-38.
63. Diamond, R. D.; Clark, R. A.; Haudenschild, C. C. J. Clin. Invest. 1980, 66 908-17.
64. Belding, M. E.; Klebanoff, S. J.; Ray, C. G. Science, 1970, 167, 195-96.
65. Jacobs, A. A.; Low, I. E.; Paul, B. B.; Strauss, R. R.; Sbarra, A. J. Infect. Immun. 1972, 5, 127-31.
66. Yong, E. C.; Kno, C. C.; Klebanoff, S. J. Am. Soc. Microbiol. Abst. Annual Meeting 1980, p. 38.
67. Chang, K. -P. J. Trop. Med. Hyg. 1981, 30, 322-33.
68. Jong, E. C.; Mahmoud, A. A. F.; Klebanoff, S.J. J. Immunol. 1981, 126, 468-71.
69. Klebanoff, S. J.; Smith, D. C. Biol. Reprod. 1970, 3, 236-42.
70. Klebanoff, S. J.; Clark, R. A. Blood 1975, 45, 699-07.
71. Clark, R. A.; Klebanoff, S. J. Blood 1977, 50, 65-70.
72. Clark, R. A.; Klebanoff, S. J. J. Clin. Invest. 1979, 63 177-83.
73. Clark, R. A.; Klebanoff, S. J.; Einstein, A. B. Blood 1975, 45, 161-70.
74. Clark, R. A.; Klebanoff, S. J. J. Clin. Invest. 1979, 64 913-20.
75. Paredes, J.; Weiss, S. J. J. Biol. Chem. 1982, 257 2738-40.
76. Klebanoff, S. J. In Phagocytic Cells; Gallin, J. I.; Fauci, A. S.; Eds.; Raven Press, New York, 1982; pp 111-62.
77. Rosen, H.; Klebanoff, S.J. J. Biol. Chem. 1977, 252, 4803-10.
78. Khan, A. U. Biochem. Biophys. Res. Commun. 1984, 122, 668-75.
79. Kanofsky, J. R.; Wright, J.; Miles-Richardson, G. E.; Tauber, A. I. J. Clin. Invest. 1984, 74 1489-95.
80. Theorell, H.; Akeson, A. Arkiv. Kemi. Mineral. Geol. 1943, 17B, No. 7.
81. Theorell, H.; Paul, K. G. Arkiv. Kemi. Mineral. Geol. 1944, 18A, No. 12.
82. Kanofsky, J. R. J. Biol. Chem. 1983, 258, 5991-93.
83. Khan, A. U., J. Am. Chem. Soc. 1983, 105, 7195-97.
84. Morris, D. R.; Hager, L. P. J. Biol. Chem. 1966, 241, 1763-68.
85. Thomas, J. A.; Morris, D. R.; Hager, L. P. J. Biol. Chem. 1970, 245, 3129-34.
86. Khan, A. U. unpublished.
87. Samuelson, B. Science 1983, 220, 568-75.
88. Corey, E. J. Experientia 1982, 38, 1259-1381.

89. Kanofsky, J. R.; Axelrod, B. <u>J. Biol. Chem</u>. 1986, <u>261</u>, 1099–104.
90. Russell, G. A. <u>J. Am. Chem. Soc</u>. 1957, <u>79</u>, 3871–77.
91. Moureu, C.; Dufraisse, C.; Dean, P. M. <u>C.R. Acad. Sci</u>. 1926, <u>198</u>, 1584–85.
92. Dufraisse, C.; Velluz, L.; Velluz, L. <u>C.R. Acad. Sci</u>. 1939, <u>208</u>, 1822–24.
93. Rigaudy, J.; Guillaume, J.; Maurette, D. <u>Bull. Soc. Chem. Fr</u>. 1971, 144–52.
94. Wasserman, H. H.; Scheffer, J. R.; Cooper, J. L. <u>J. Am. Chem. Soc</u>. 1972, <u>94</u>, 4991–96.
95. Wilson, T. <u>Photochem. Photobiol</u>. 1969, <u>10</u>, 441–44.
96. Wilson, T.; Khan, A. U.; Mehrotra, M. M. <u>Photochem. Photobiol</u>. 1986, <u>43</u>, 661–62.
97. Chou, P. T.; Frei, H. <u>Chem. Phys. Lett</u>. 1985, <u>122</u>, 87–91.
98. Corey, E. J.; Mehrotra, M. M.; Khan, A. U. <u>J. Am. Chem. Soc</u>. 1986, <u>108</u>, 2472–73.

Chapter 5

Singlet Oxygen Quantum Yields

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The lowest excited state of molecular oxygen (also known as dioxy-
gen) has the spectroscopic notation $O_2(^1\Delta_g)$. Its v=0 vibrational
level lies 7880 cm^{-1} (1eV; 23kcal/mol) above the v=0 level of the
molecular ground state $(^3\Sigma_g^-)$. The $^1\Delta_g \longrightarrow {}^3\Sigma_g^-$ transition and its
inverse are strongly forbidden for electric dipole radiation in the
isolated molecules -- at zero pressure in the gas phase the radiat-
ive lifetime of $O_2(^1\Delta_g)$ is calculated to be 45 mins.([1]). This
property is apparently phase dependent since a value of 4s has been
reported in carbon tetrachloride ([2]). This forbiddeness of the op-
tical transition makes generation of $O_2(^1\Delta_g)$ by direct-photon ab-
sorption very difficult to accomplish although quantities
sufficient to allow kinetic studies in Freons have been produced by
irradiation of high pressures of O_2 in Freon solution with 1.064 μm
radiation from a high power Nd: YAG laser ([3]).
 The extremely low probability of the radiative transition has
several consequences, the one that is pertinent to this account
concerns the use of indirect methods of producing $O_2(^1\Delta_g)$ for quan-
titative kinetic studies. Such investigations are generally per-
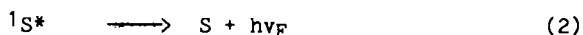formed in one of two ways.
 (i) Singlet oxygen is formed at a constant rate by applica-
tion of some perturbation that operates continuously. The progress
of reactions are followed by measuring the yields of chemical prod-
ucts or other effects as a function of time over which the pertur-
bation is continued.
 (ii) Singlet oxygen is formed by a short high, intensity burst
of the perturbing effect such that the concentration of singlet
oxygen produced is sufficient to be followed, either directly or
indirectly, in time-resolved experiments.
 Both kinds of experiment are capable of yielding kinetic data
of interest such as natural lifetimes, reaction rate constants,
quenching rate constants and so forth.
 The perturbation effect most often employed is that of photo-
excitation of a sensitizer. This act forms upper singlet sensi-
tizer states that can undergo inter-system crossing to triplet
states (Reactions 1-4).

$$S + h\nu \longrightarrow {}^1S* \tag{1}$$

$$^1S* \longrightarrow S + h\nu_F \tag{2}$$

$$^1S* \longrightarrow {}^3S* \tag{3}$$
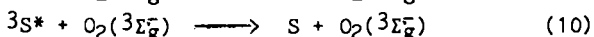
$$^1S* \longrightarrow S \tag{4}$$

Subsequently, the triplet state can decay according to Reactions (5) and (6)

$$^3S* \longrightarrow S + h\nu_p \tag{5}$$

$$^3S* \longrightarrow S \tag{6}$$

In the above scheme $h\nu_F$ and $h\nu_p$ refer to the radiative processes denoted as fluorescence (Reaction 2) and phosphorescence (Reaction 5) respectively. For most sensitizers in fluid media the phosphorescent channel contributes only minimally to the total decay of the triplet state population.(4)

When oxygen is dissolved in the solvent (aerated solvents are normally employed) the following additional reactions are possible.

$$^1S* + O_2(^3\Sigma_g^-) \longrightarrow {}^3S* + O_2(^1\Delta_g) \tag{7}$$

$$^1S* + O_2(^3\Sigma_g^-) \longrightarrow S + O_2(^1\Delta_g) \tag{8}$$

$$^1S* + O_2(^3\Sigma_g^-) \longrightarrow S + O_2(^3\Sigma_g^-) \tag{9}$$

$$^3S* + O_2(^3\Sigma_g^-) \longrightarrow S + O_2(^3\Sigma_g^-) \tag{10}$$

$$^3S* + O_2(^3\Sigma_g^-) \longrightarrow S + O_2(^1\Delta_g) \tag{11}$$
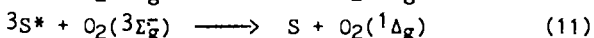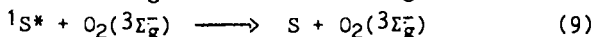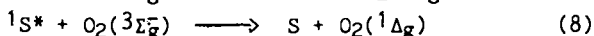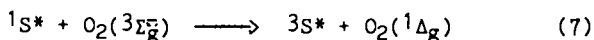
Clearly Reaction (7) is spin-allowed but is only possible when $(E_S-E_T > E_\Delta)$. Reaction (8) is spin forbidden and Reaction (9) has severe Franck-Condon restrictions in that the energy of $^1S*$ has to be dissipated into vibrational modes. Similar restrictions apply to Reaction (10) which is in competition with Reaction (11), the singlet oxygen-producing channel from sensitizer triplet states.

RATIONALE FOR MEASURING SINGLET OXYGEN QUANTUM YIELDS

The reason why there should be so much interest in determining quantum yields of singlet oxygen fall into two major categories, one concerning fundamental photophysics, the other concerning applications of photosensitized oxidation.

The photophysics requirement concerns expanding our knowledge about the interactions of the sensitizer excited states with oxygen as summarized in Reactions (7) through (11) above. Quantitative measurement of the yields of $O_2(^1\Delta_g)$ produced from molecular singlet states and molecular triplet states aids in assessing the amount that a particular reaction-channel contributes to the overall deactivation. Information on how the yields vary with influences such as sensitizer structure, state energy, nature of solvent and so on is important in providing mechanistic information. The quenching of excited states by oxygen is such a well know process that it may be surprising to many to learn that it is so little understood.

The area of photosensitized oxidations is very wide, ranging over all systems where the combined action of visible light, an absorbing molecule or residue, and molecular oxygen can result in photochemical damages (5). This combination has impact in such diverse places as the textile industry, the cancer clinic and the plant leaf. Textile technologists are concerned with the photofading and photodestruction of fibers that have been dyed with visible light absorbing pigments; in the cancer clinic trials are proceeding in which porphyrin doped tumors undergo necrosis when irradiated with red light; the photosynthetic apparatus of green plants are ideally suited for producing singlet oxygen from photoexcited chlorophyll molecules -- the presence of carotenoids offers non-damaging physical modes of deactivating chlorophyll triplet states. Processes such as these, together with the action of light-activated pesticides, fall under the general heading of photodynamic action, ie damage incurred in a biological system through the photosensitized oxidation mechanisms (6). There is a wealth of evidence indicating the involvement of singlet oxygen as a primary reactive species in one class of photodynamic action. Thus the more quantitative information that we can obtain about the yields of singlet oxygen in photodynamic circumstances, then the greater our opportunity for understanding the detailed mechanism and altering, (enhancing or diminishing, according to the requirements) its effects.
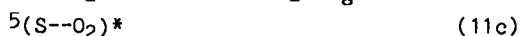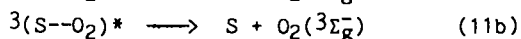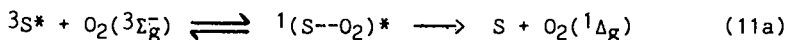
SINGLET STATE SOURCES

A consideration of Reactions (7), (10) and (11) shows that for sensitizers having a sufficient S-T energy difference, each $^1S*$ state (ie each photon) will give rise, in the limit, to two $O_2(^1\Delta_g)$ molecules -- Reaction (7) followed by Reaction (11), ie the singlet oxygen quantum yield (Φ_Δ) can approach 2.0. Several researchers (7-9) have investigated such processes and evidence for Φ_Δ values greater than unity has been obtained. Substituted anthracenes and higher homologues show this effect. Of course, the limiting quantum yield is rarely achieved simply because at oxygen concentrations attainable in O_2-saturated organic solvents (typically $10^{-2}M$), the product $k_7[O_2]$ is usually unable to outweigh the sum $(k_2 + k_3 + k_4)$ ie, singlet states are generally lost to the unimolecular decay modes with approximately similar efficiency to that with which they are quenched by oxygen.

TRIPLET STATE SOURCES

With molecules that have $E_S - E_T < E_\Delta$, Reaction (7) is not energetically feasible and since Reaction (8) is spin-forbidden, the only source of $O_2(^1\Delta_g)$ from excited states of many systems is through the triplet manifolds via oxygen quenching. In many molecular systems, oxygen quenching of triplet states is the only process for singlet oxygen production.

Reaction (11) above can be expanded into the set (Reactions 11a-c) as below and in quantitative terms, competition between the set determines the rates of $O_2(^1\Delta_g)$ formation and the quantum yields.

$$^3S^* + O_2(^3\Sigma_g^-) \;\rightleftharpoons\; {}^1(S\text{--}O_2)^* \;\longrightarrow\; S + O_2(^1\Delta_g) \qquad (11a)$$

$$^3(S\text{--}O_2)^* \;\longrightarrow\; S + O_2(^3\Sigma_g^-) \qquad (11b)$$

$$^5(S\text{--}O_2)^* \qquad (11c)$$

The species $^{1,3,5}(S\text{--}O_2)^*$ represents the transition state between reactants and products. The mutual triplet multiplicity of the reactants conveys nine possible degenerate spin sub-states within the transition state; the singlet and triplet entities have energetically reachable product states but the quintets have not and they must return to reactant states.

The consequence of this is that the rate constant for singlet oxygen formation will not be larger than one ninth of the value of the rate constant for diffusion-limited quenching in the medium (10). That the measured rate constants for oxygen quenching of the triplet states of many molecules fall close to this one ninth of diffusion control value (near $2 \times 10^9 \ 1 \ mol^{-1}s^{-1}$) has been taken as an indication (10) that in such instances the singlet channel (Reaction 11a) provides the only triplet deactivation mechanism, thereby leading to $O_2(^1\Delta_g)$ quantum yields of unity. However, more detailed observations show (11-13) this is not necessarily true and that the system is more complicated than first thought.

The yield of $O_2(^1\Delta_g)$ formed from a molecular triplet state involves a competition between Reactions (10) and (11) and the parameter S_Δ was coined (11) to describe this where

$$S_\Delta = k_{11}/(k_{11} + k_{10})$$

or, S_Δ is the fraction of triplet quenchings by oxygen that leads to singlet oxygen. Thus in a photochemical system that involves $O_2(^1\Delta_g)$ formation via sensitizer triplet states only, we see that

$$\Phi_\Delta = S_\Delta \cdot \Phi_T$$

where Φ_T is the triplet quantum yield and Φ_Δ is the overall singlet oxygen quantum yield. Therefore, a quantitative discussion of singlet oxygen quantum yields must always take into account the involvement of the independent variables S_Δ and Φ_T.

SINGLET OXYGEN YIELD MEASUREMENTS

Measurements of singlet oxygen have been carried out using both steady-state and pulsed illumination methods, but it has never proved an easy molecule to measure largely because it is not amenable to optical absorption spectroscopy using conventional instruments. Earlier means of detection employed chemical traps, ie, molecules that react with $O_2(^1\Delta_g)$ resulting in loss of starting material (M) and/or production of a relatively stable peroxy product, or a thermally-activated decay product thereof.

$$M + O_2(^1\Delta_g) \;\longrightarrow\; MO_2 \;\longrightarrow\; product \qquad (13)$$
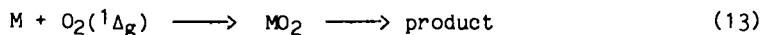
Perhaps the most widely-used substrate (M) has been diphenyl-isobenzofuran (DPBF) which, on reaction with singlet oxygen loses its characteristic yellow color. Initially DPBF was employed (14-17) in situations where kinetic data were being evaluated. Later the extent of bleaching became employed in yield evaluations (11,18-20). Singh et al (21) have outlined the potential difficulties in using DPBF as a quantitative probe for singlet oxygen in continuous illumination conditions. In time-resolved experiments these problems are less severe but caution is always necessary on account of the molecule's high photosensitivity. Like DBPF, some condensed polycyclic aromatic hydrocarbons (rubrene, 9,10 diphenyl-anthracene) form endoperoxides with $O_2(^1\Delta_g)$ - a reaction that removes extended chromophores and leads to bleaching. These molecules have also been used for quantitative yield evaluations (7-9,22,23).

In determining the yield of singlet oxygen from a series of rose bengal derivatives, Neckers et al have employed 2,3 diphenyl-dioxene as a probe for $O_2(^1\Delta_g)$. This species is oxidized to an acyclic ester which was quantified gas chromatographically (24,25). Another method has been to use para-nitrosodimethylaniline (RNO) - a green-colored molecule - as a reactive probe for transannular peroxides (MO_2) formed from singlet oxygen reaction with imidazoles. Again the coloration of M is lost and this property is followed quantitatively to result in Φ_Δ values (23,26). Those chemical probe systems that depend on a simple color change can be, and have been employed in both steady-state and time-resolved experimentation. Of course in using reactive monitor molecules (DPBF, rubrene, etc.) for quantum yield measurement it is important to know what fraction of the deactivating encounters leads to substrate loss. For DPBF this fraction is apparently unity (27).

In recent years researchers have been using such indirect chemical probe techniques less, and newly-developed direct spectroscopic methods more. The use of chemical probes can be subject to problems arising out of uncertainties in reaction mechanisms with different sensitizers. Also they require extreme care in exclusion of extraneous light. The advent of fast response detectors with infrared sensitivity and high bandwidth, high gain amplifiers has given the capability of detecting the very weak luminescence at 1.269 μm (Figure 1) resulting from the $^3\Sigma_g^- \longleftarrow {}^1\Delta_g$ transition in oxygen. The forbiddeness of this transition results in luminescence quantum yields being very low. The earliest work in this area was carried out by Russian workers who pioneered luminescence detection in both c.w. (28) and time-resolved modes (29,30). These early efforts were rapidly followed by activity in the U.S. where red-sensitive photomultiplier detectors were replaced by infrared detecting photodiodes backed-up by high gain amplifiers for continuous wave (c.w.) (31) and time-resolved work (32-35). This technology has revolutionized $O_2(^1\Delta_g)$ detection because of its directness, precision, convenience and rapidity. The vast majority of research on $O_2(^1\Delta_g)$ using infrared luminescence techniques has concerned kinetic studies but these methods are also applicable to quantum yield studies once the proper calibration has been carried out. This calibration process is essential and it is described in some detail below.
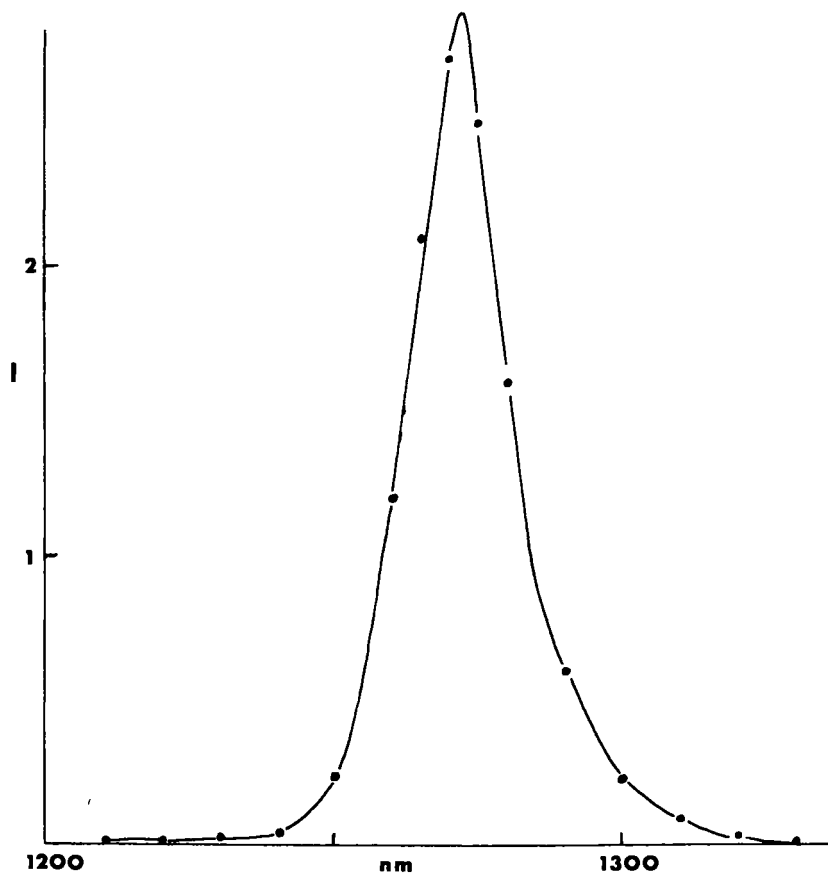
Figure 1. Luminescence from singlet oxygen in aerated benzene
solution. The sensitizer was 2-acetonaphthone excited at 365
nm. The bandwidth at FWHM is 20 nm. Taken with the apparatus
(Rodgers, M.A.J., to be published) shown in figure 2.

Another physical technique that has recently been developed is the use of thermal lensing. This depends upon the deactivation of a population of excited molecules by non-radiative modes, ie, the excitation energy is released to the surrounding medium as thermal energy. This heat release, if rapid enough, ie after laser pulse excitation, produces local changes in temperature, density and refractive index in the medium. Thus the sample behaves momentarily as a diverging lens and a carefully aligned optical system can be set up to probe the transient lens and the resulting signal contains information on both the kinetics of the non-radiative decay channels and on relative magnitudes of the contributions from the various decay modes. Since singlet oxygen decays almost exclusively non-radiatively, it is particularly well-suited for thermal lensing studies. Fuke, et al (36) first used this technique for kinetic measurements and it has recently been refined and quantified for yield measurements by Rossbroich et al (37). The method offers the capability of measuring Φ_Δ and Φ_T for photoexcited molecules by the relatively straightforward expedient of measuring the slow heat contribution in aerated and de-aerated solutions respectively (37).

APPARATUS FOR INFRARED LUMINESCENCE MEASUREMENT

Fuller descriptions of the infrared detection methodology occur elsewhere in this volume (38). Briefly presented here are two systems extant in the author's laboratory for c.w. and time-resolved quantum yield measurements.

C.W. EXCITATION

This is shown schematically in Figure 2 and is a development of systems used by Khan (39), Kanofsky, (40) and Hall and Chignell (Photochem. Photobiol., in press). Solutions in the 10mm x 10mm sample cuvette are irradiated with light from a 100W Hg arc filtered through 10 cm of water and a heat absorbing filter (Schott KG-3). This combination transmits mercury lines at 365 nm and above with ca 90% efficiency but stops radiation above 1000 nm with high efficiency. Luminescence is collected at right angles by an Anaspec Cassegrain mirror system (f/1.0) which conveys the light to a monochromator (Oriel) with a 600 lines per mm, 1.0 μm blaze grating. Monochromated radiation from the output slit is focussed onto a 5mm^2 germanium crystal PN detector coupled to a transimpedance preamplifier (North Coast Optical Systems and Sensors). Both detectors and preamplifier are cooled to 77 K. This system has a responsivity of 5 x 10^9 V/W. THe detector is covered by a 5 mm thick disc of AR-coated high purity silicon metal acting as an 1100 nm cut-off filter. The excitation beam is chopped at 100 Hz and the output from the preamplifier is fed to a lock-in amplifier (Princeton Applied Research 124A) incorporating a multirange volt meter. A spectrum of singlet oxygen measured with this instrument is shown in Figure 1.
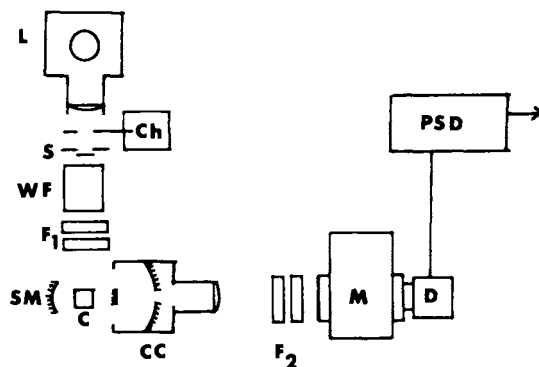
Figure 2.   Infrared emission spectrophotometer schematic.
L: 100W Hg arc with lens assembly; S: shutter; Ch: variable
frequency chopper; WF: 10 cm water filter; F_1: lamp filters in-
cluding KG3 heat filter and Hg line filter; C: 10 mm sample
cuvette; SM; spherical mirror; CC: Cassegrain f/1 light collec-
tor; F_2 cutoff and order blocking filters; M: monochromator;
D: Germanium detector and amplifier; PSD: phase sensitive de-
tector.

PULSED EXCITATION

The current version of this apparatus (Figure 3a) varies only mar-
ginally from that described earlier (35,41). The excitation beam is
a 10 ns pulse from Q-switched Nd:YAG laser operating in conjuction
with harmonic generators to produce light at 532 nm or 355 nm.
Attenuation is arranged by placing a quartz diverging lens (-5 mm)
about 12 inches from the cuvette position and interposing neutral
density filters for further decreases. The 10 mm x 10 mm sample
cuvette is held closely against a disc of AR-coated silicon metal
(at 90 degrees to the input face) immediately behind which is
placed a reverse-biased germanium photodiode (Judson J16) operating
photo-conductively. The photodiode output is taken to a preampli-
fier (Judson) with a 500Ω output impedance. A second amplifier
with 10KΩ input impedance and 50Ω output impedance provides a fur-
ther gain of 10 and impedance matching to the 50Ω input of a Bior-
mation 8100 waveform digitizer. The input circuitry of the Judson
amplifier has been modified for switchable load resistances in the
range 50Ω to 1KΩ. The cuvette, detector and preamplifier system
are contained in an aluminum enclosure with minimum-sized entrance
and exit holes for the laser beam. This prevents RF interference
from the laser lamps and Q-switch. The apparatus has been con-
structed such that photodetectors of different sizes may be used.
Smaller area detectors allow improvements in signal rise time, up
to a limit set by the preamplifier analog bandwidth (15 MHz). Rise
times of 60 ns have been obtained with this apparatus. Figure 3b
shows some typical data obtained with this device.

QUANTUM YIELD MEASUREMENTS USING INFRARED LUMINESCENCE

Measurement methods based on light absorption are relative and
therefore have in-built calibrations. This is not so for emis-
sion-measuring systems which measure photons absolutely and thus,
in quantitative yield measurements, account has to be taken of
factors such as light collection geometry, monochromator throughput
efficiency and the detector response with wavelength. In short,
the instrument must be calibrated. This can most expeditiously be
accomplished by employing reference systems of known luminescent
quantum yield. For singlet oxygen measurements this reference may
have been obtained either through photochemical yield measurements
or from infrared luminescent measurements. Our approach has been a
combination of the two.
      The absolute standard that we developed arises out of measure-
ments of the singlet oxygen luminescence signal (I_Δ) immediately
after a laser pulse measured as a function of laser energy (Gorman,
A.A. et al, submitted for publication) The chemical system em-
ployed was a solution of benzophenone (concentration such that A_{355}
= 0.5) and naphthalene, N, (0.1M), or biphenyl, B, (0.1M), or
fluorene, F, (0.1M) in either aerated benzene or aerated cyclohex-
ane. Under these conditions the only light-absorbing species is
benzophenone which, on excitation, produces its triplet state with
unit quantum yield. This then undergoes energy transfer to the
naphthalene, etc., which is present at 0.1M to ensure 100% energy

a   CUVETTE

SILICON FILTER

GERMANIUM DIODE

FROM
LASER

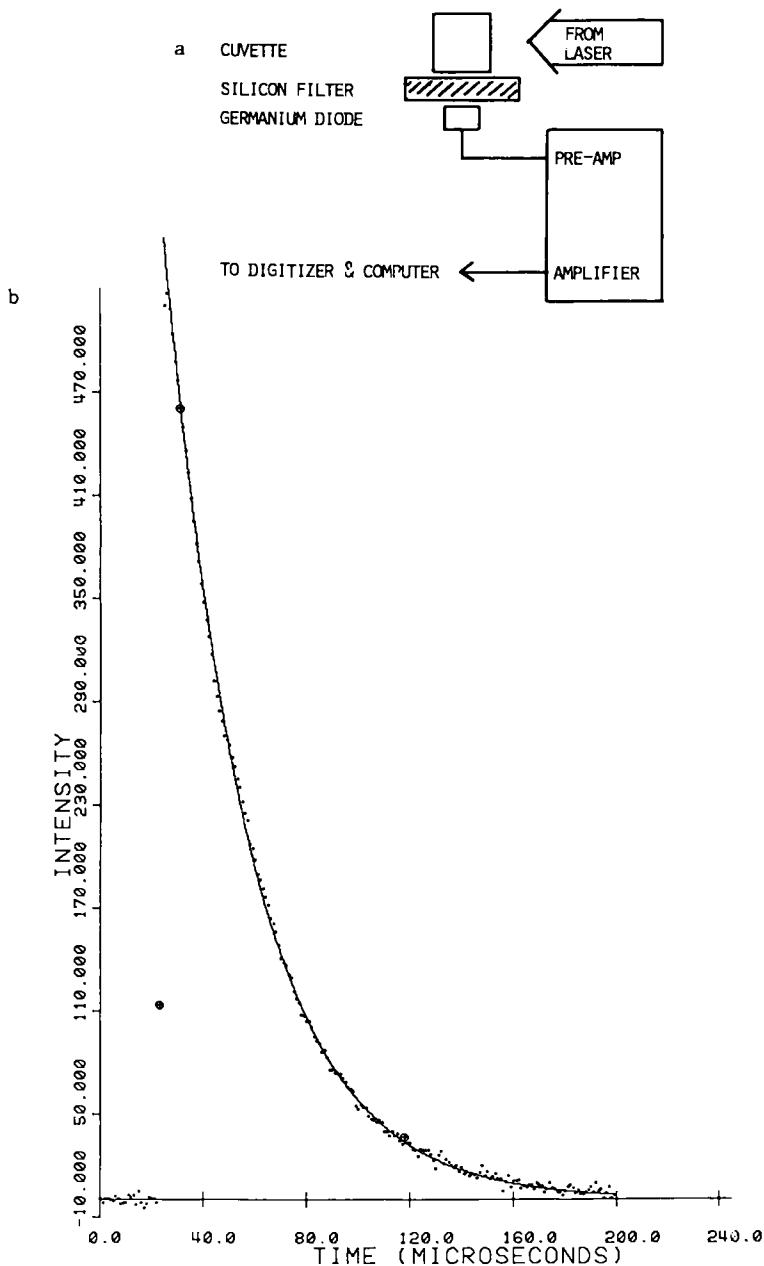PRE-AMP

TO DIGITIZER & COMPUTER

AMPLIFIER

b

Figure 3. (a) Schematic of time-resolved infrared luminescence apparatus used for obtaining intensity-time profiles of singlet oxygen emission as shown in (b), which is such a signal obtained from a 355 nm-irradiated, aerated benzene solution of 2-acetonaphthane.

transfer, whence hydrocarbon triplet states are formed with unit efficiency. This process is complete in less than 10^{-7}s. In aerated solvent, all hydrocarbon triplet states are quenched by oxygen and, since the conditions have been selected such that $\Phi_T = 1.0$, we have

$$\Phi_\Delta = S_\Delta \cdot \Phi_T = S_\Delta$$

A plot of I_Δ <u>vs</u> laser energy has a slope that is directly proportional to S_Δ and as Figure 4 shows, in benzene (upper half) the slopes for N, F and B are distinctly different and hence the S_Δ values differ. In cyclohexane, however (lower half), it is clear that N, F and B have a common slope and therefore a common S_Δ value.

That three dissimilar molecules have a common S_Δ strongly implies that the common value is unity. This was confirmed by carefully measuring the concentration of $N(T_1)$ produced immediately after a 355 nm laser pulse into a cyclohexane solution containing both benzophenone and naphthalene as above, then, under identical conditions measuring the concentration of diphenylisobenzofuran bleached by the $O_2(^1\Delta_g)$ generated from the population of N (T_1). This confirmed that for naphthalene in cyclohexane, $S_\Delta = 1.0$. A similar experiment was used to show that $S_\Delta = 0.55$ for naphthalene in benzene. Two other series of experiments showed that in acetonitrile $S_\Delta = 1.0$ for naphthalene and, in a medium composed of sodium dodecyl sulfate (SDS) micelles in D_2O containing benzophenone and naphthalene, again $S_\Delta = 1.0$ was obtained. In this way we now have absolute standards for S_Δ and further, we have absolute standards for Φ_Δ where ketone triplets with $\Phi_T = 1.0$ are used as primary sensitizers (Gorman, A.A. <u>et al</u>, submitted for publication).

As an example suppose we have a molecule X of unknown S_Δ and Φ_Δ. If X absorbs significantly at 355 nm its Φ_Δ can be determined by measuring I_Δ^X as a function of laser energy for a solution of X in cyclohexane (or any other known solvent) and doing the same for a standard benzophenone-naphthalene solution (in the same solvent). The ratio of the slopes of the two plots is identical to the ratio of the Φ_Δ values for X and N and since the latter is unity Φ_Δ^X can be calculated.

Otherwise, where X does not absorb at 355 nm and $E_T^X < E_T^{BP}$, then S_Δ^X can be measured I_Δ from optically-matched solutions containing (in the same solvent), (a) benzophenone and naphthalene, and (b) benzophenone and X. Then we see that

$$\frac{I_\Delta^X}{I_\Delta^N} = \frac{\Phi_\Delta^X}{\Phi_\Delta^N} = \frac{S_\Delta^X \cdot \Phi_\Delta^X}{S_\Delta^N \cdot \Phi_\Delta^N}$$

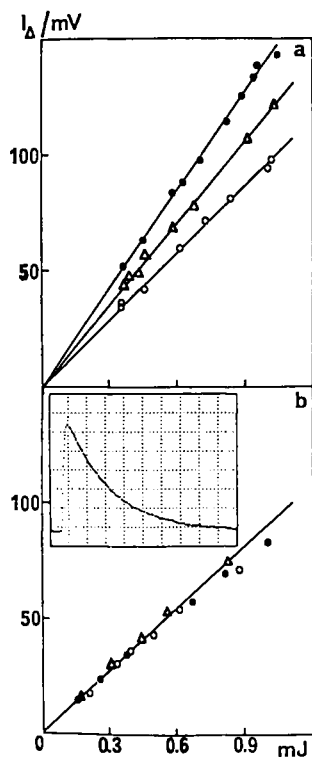but $\Phi_T^X = \Phi_T^N = \Phi_T^{Bp}$ since complete energy transfer occurs, thus:

Figure 4. Infrared emission intensity extrapolated to t=0 (I_Δ) versus laser energy for (a) aerated benzene solutions of p-methoxy acetophenone with 0.1M naphthalene (Δ), biphenyl (o) or fluorene (o) and (b) corresponding plot for cyclohexane solutions. Insert shows typical intensity-time profile in latter with τ = 23.5 s.

$$\frac{I_\Delta^X}{I_\Delta^N} = \frac{S_\Delta^X}{S_\Delta^N}$$

and since S_Δ^N is known (in benzene, cyclohexane, acetonitrile and SDS micelles) then S_Δ^X is calculated. Having determined S_Δ we can compute ϕ_Δ^X when ϕ_T^X is known.

An important point to remember in experiments such as these in which the luminescence intensity is used as a measure of $O_2(^1\Delta_g)$ concentration is that the intensity of luminescence at any time is a product of the number of emitting states and their radiative probability. This last, usually denoted by the radiative rate constant (k_R) for the transition $^1\Delta_g \longrightarrow {}^3\Sigma_g^-$ probably varies from solvent to solvent. Thus measurements of unknown and standard must employ a common solvent.

VALUES OF ϕ_Δ AND S_Δ FOR A VARIETY OF SENSITIZERS

A summary of S_Δ values for a number of photosensitizers in a variety of solvents is presented in Table I. These were obtained using the infrared luminescence measurements as described in the preceeding section (44). For many of these systems the literature contains ϕ_T values thus enabling a calculation of the ϕ_Δ that would result from direct excitation of the photosensitizer. Recently, using thermal lensing (37). Rossbroich et al have confirmed the anthracene values in Table I and have experimentally shown that for this molecule $\phi_T = 0.78$. They also measured ϕ_Δ values for meso-tetraphenylporphine and its zinc derivatives finding values of 0.58 and 0.73 respectively. Using laser flash photolysis and the DPBF bleaching method Chattopadhyay et al (19,20) have determined S_Δ values for a series of aromatic ketones and some polyenes. These are presented in Table II. The furocoumarins are a class of molecules that have significance in photobiology and ϕ_Δ values for a series of such molecules have been measured (42) using the infrared luminescence method. These data are collected in Table III. Some furocoumarins show very weak singlet oxygen production. This appears to arise mainly from low intersystem crossing yields (Table III.)

All the evaluations in Tables I through III have been obtained using ultra-violet excitation, whereas most photodynamic interest centers around visible-absorbing dyes such as xanthenes, porphyrins, phthalocyanines, and so forth. Although visible-absorbing dyes to some extent also absorb u.v. radiation and are thus measurable by those methods used for aromatic ketones, etc., uncertainties exist as to whether wavelength effects can occur. The techniques that have been used for uv-absorbing molecules are in principle translocatable to the visible. Some restrictions occur in practice, however. For example, those methods that rely on the transient bleaching of DPBF[14] or anthracene dipropionic acid (21) may not be useable for sensitizers (eg porphyrins) that absorb light strongly near 400 nm where these probes are also absorbing.

Table I. S_Δ Values for U-V Absorbing Sensitizers
in Several Solvents

Sensitizer	SOLVENTS			
	Cyclo-hexane	Benzene	Aceto-nitrile	Aq.SDS Micelles
naphthalene (a)	1.0	0.55	1.0	1.0
biphenyl (a)	1.0	0.42	---	1.0
fluorene (a)	1.0	0.91	1.0	---
acridine (b)	---	1.0	---	---
benzophenone (b)	---	0.29	---	---
anthracene (c)	---	0.8	---	---
fluorenone (c)	---	0.8	---	---
2-acetonaphthone	0 .93 (d)	0.7 (b)	---	---
cyclopentadiene (d)	---	0.58	---	---
cyclohexadiene (d)	0.66	0.39	---	---
cycloheptatriene (d)	0.89	0.69	---	---
tetramethyl-butadiene (d)	---	0.45	---	---
neo-allocimene (d)	0.56	0.43		
trans-stilbene (e)	---	0.18	---	---
ergosterol (f)	---	0.78	---	---

(a) Gorman, A.A. <u>et al</u>, to be published
(b) reference 13
(c) reference 46
(d) Gorman and Rodgers, to be published
(e) reference 47
(f) Gorman, A.A.; Hamblett, I; Rodgers, M.A.J.
Photochem. Photobiol. In press

Table II. Φ_Δ Values for Aromatic Ketones, Polyene
and other Sensitizers[a]

Sensitizer	Benzene	Cyclo-hexane	Aceto-nitrile	Methanol
acetophenone	0.35	nrr	0.52	cnc
p-methoxyacetophenone	0.27	ccc	0.42	arr
p-cyanoacetophenone	0.49	crn	0.76	rcn
bz*	0.39	npr	0.37	nnr
p,p'-bis(N,N-dimethylamino)bz*	0.41	nnc	0.35	nnn
p,p'-dimethoxy bz*	0.34	ncr	0.40	nnn
p-fluorobz*	0.43	ncr	0.44	arr
p-trifluoromethylbz*	0.42	nrn	0.54	rrn
pyrene-1-aldehyde	0.68	0.87	ccn	ncn
all-trans-retinal	nrc	0.66	ncr	0.20
β-apo-14'-carotenal (b)	nrc	0.48	crn	ncr
c₁₇ aldehyde (c)	crc	0.72	ncr	0.42

* bz = benzophenone

(a) references 19 and 20, all using DPBF bleaching and transient spectroscopy.

(b) immediate higher homologue of retinal (C_{20} aldehyde).

(c) immediate lower homologue of retinal.

Table III. S_Δ and Φ_Δ Values for some Furocoumarins in Benzene(a)

Sensitizer	S_Δ	Φ_Δ
psoralen	0.34	0.012
pseudopsoralen	0.57	0.026
5-methoxypsoralen	0.32	0.021
8-methoxypsoralen	0.40	0.0044
5,8-dimethoxypsoralen	0.10	0.0040
4,5,8-trimethylpsoralen	0.41	0.084
3-carbethoxypsoralen	1.0	0.30
3-carbethoxypseudopsoralen	0.96	0.49

(a) reference 42: using infrared luminescence with Φ_T measurement by transient absorption.

Also the infrared luminescence method has not been calibrated for
visible wavelength absorbers but this is currently underway in the
author's laboratory.

Most quantum yield work on the xanthene dyes (rose bengal,
eosin, etc.) has been carried out using the steady state tech-
niques. Thus Gandin et al (23) used both ADPA bleaching and the
RNO method to evaluate Φ_Δ values for a series of substituted fluo-
resceins in water (Table IV). Lamberts and Neckers (24) used the
photosensitized oxidation of 2,3 diphenyl-p-dioxene for measuring
Φ_Δ values for a series of rose bengal derivatives (Table V).
Paczkowski and Neckers (25) used the same method for evaluating Φ_Δ
for a series of rose bengal-tagged polymers with different degrees
of loading.

Because of the interest in porphyrins in photodynamic tumor
therapy, many porphine derivatives have been subjected to experi-
mental evaluations for singlet oxgyen formation. Reddi et al (43)
found Φ_Δ varied between 0.27 and 0.65 for hematoporphyrin in media
varying from aqueous micelles to aqueous methanol. These experi-
ments indicated that S_Δ varied with solvent more than did Φ_T. More
recently (44) the infrared luminescence method has been used to
evaluate Φ_Δ of several porphyrins (355 nm excitation) in benzene
solution (Table VI). Verlhac et al (26) have used the RNO method
to measure Φ_Δ values for a series of water-soluble porphyrins and
their metallo-derivatives (Table VII).

The contents of this section represent the majority of the
data that is currently available on singlet oxygen quantum yields
(Φ_Δ) and on its efficiency of formation from a sensitizer trip-
let-state (S_Δ). A scrutiny of the Tables shows clearly that photo-
excitation of a sensitizer leads to a quantum efficiency of singlet
oxygen production, the order of magnitude of which depends predomi-
nantly upon Φ_T. This is because S_Δ values lie between 0.3 and 1.0
(the only molecule outside this range is 5,8 dimethoxypsoralen
which has S_Δ = 0.1 -Table IV). Thus the rule of thumb must be:
when selecting a sensitizer for photodynamic work look for one with
high Φ_T.

Finally, recalling that the S_Δ parameter informs on the nature
of interaction between the molecular triplet state and oxygen, we
need to ask what factors are causing S_Δ values to vary in the broad
range 0.3 to 1.0? There is no definitive answer yet to this ques-
tion, but there are indications that for some aromatic ketones (eg
benzophenone, acetophenone) the collision complex in reaction
11(a-c) can also find a bond-forming route to the ground state,
perhaps involving a biradical species. More evidence still needs
to be collected to substantiate this and to determine whether this
putative chemical channel exists for other sensitizers.

Table IV. Φ_Δ Values from Xanthene Dyes in Aqueous Solution[a]

Xanthene [b]	Φ_Δ
FlI_4Cl_4 [c]	0.75
$FlBr_4Cl_4$	0.65
FlI_4 [d]	0.63
$FlBr_4$ [e]	0.57
$FlBr_2(NO_2)_2$	0.52
FlI_2	0.48
$FlBr_3$	0.44
$FlBr_2$	0.42
$2',7'FlCl_2$	0.07
$4',5'FlCl_2$	0.07
$FlCl_4$	0.05
$Fl.$	0.03

(a) reference 23: by steady-state techniques using ADPA bleaching and RNO method.
(b) Fl indicates fluorescein skeleton
(c) rose bengal   (d) erythrosin B   (e) eosin Y

Table V. Φ_Δ Values Obtained for a Series of Derivatives
of Rose Bengal(a)

R_1 residue(b)	R_2 residue(b)	Φ_Δ	
		Methanol	Dichloromethane
Na	Na	0.76	--
Ethyl(Et)	H	0.73	0.61
Et_3NH	Et_3NH	0.72	0.48
$PhCh_2$	Et_3NH	0.74	0.67
Et	Et_3NH	0.74	0.71
Et	Acetyl	--	0.61

(a) reference 24: steady state illumination, diphenyl-p-dioxene photocon-
version.
(b) R_1 is at carboxylate; R_2 is at phenoxide. See original paper (24) for
structural features.

Table VI. S_Δ and Φ_Δ Values for some Porphyrins
in Benzene Solution[a]

Porphyrin	S_Δ	Φ_Δ
protoporphyrin dimethylester	0.71	0.57
hematoporphyrin dimethylester	0.70	0.50
mesoporphyrin dimethylester	0.70	0.57
deuteroporphyrin dimethylester	0.78	0.56
photoprotoporphyrin dimethylester	0.74	0.49

(a)   reference 44: by infrared luminescence measurements.

Table VII. Φ_Δ Values for Water-Soluble Porphyrins and
Metallo Derivatives in Water at pH=7[a]

Porphyrin	Φ_Δ
meso-tetra (4-N-methylpyridyl) porphine (TMPy)	0.74
meso-tetra (4-carboxyphenyl) porphine	0.58
meso-tetra (4-sulfonatophenyl) porphine	0.62
meso-tetra (4-N,N´,N´´-trimethylaminophenyl) porphine	0.77
hematoporphyrin	0.22
zinc-TMPyP	0.88
magnesium - TMPyP	0.69
cadmium - TMPyP	0.75
palladium - TMPyP	0.12
copper (II) - TMPyP	$<10^{-3}$
cobalt - TMPyP	$<10^{-3}$
manganese - TMPyP	$<10^{-3}$

(a)   reference 26: using RNO method.

Literature Cited

1.   Arnold, S.J.; Kubo, J.; Ogryzlo, E.A. In Advan. Chem. Ser.,
     77, 133, 1968.
2.   Krasnovsky, A.A. Chem. Phys. Lett., 1981, 81, 443.
3.   Matheson, I.B.C.; Lee, J. Chem. Phys. Lett. 1972, 14, 350.
4.   Birks, J.B. Photophysics of Aromatic Molecules; Wiley: New
     York, 1970.
5.   Spikes, J.B. In The Science of Photobiology; Smith, K., Ed.;
     Plenum: New York, 1977.
6.   The Science of Photomedicine; Regan, J.D.; Parrish, J.A.,
     Eds.; Plenum: New York.
7.   Brauer, H.D.; Acs. A.; Drew, W.; Gabriel, R.; Ghaeni, S.;
     Schmidt, R. J. Photochem. 1984, 25, 475.
8.   Stevens, B.; Ors, J.A. J. Phys. Chem. 1986, 80, 2164.
9.   Wu, K.C.; Trozzolo, A.M. J. Phys. Chem., 1979, 83, 3180.
10. Gijzeman, O.L.J.; Kaufman, F.; Porter, G. J. Chem. Soc.
     Faraday Trans. II, 1973, 69, 708.
11. Gorman, A.A.; Lovering, G.; Rodgers, M.A.J. J. Amer. Chem.
     Soc. 1978, 100, 4527.
12. Garner, A.; Wilkinson, F. In Singlet Oxygen; Ranby, B.;
     Rabek, J.F., Eds.; Wiley: New York, 1978; p. 48.
13. Gorman, A.A.; Hamblett; I.; Rodgers, M.A.J. J. Amer. Chem.
     Soc., 1984, 106, 4679.
14. Adams, D.R.; Wilkinson, F. J. Chem. Soc., Faraday Trans. II,
     1972, 68, 586.
15. Merkel, P.B.; Kearns, D.R. Chem. Phys. Lett. 1971, 12, 120.
16. Young, R.H.; Brewer, D.; Keller, R.A. J. Amer. Chem. Soc.
     1973, 905, 375.
17. Gorman, A.A.; Lovering, G.; Rodgers, M.A.J. Photochem. Photo-
     biol. 1976, 23, 299.
18. Ramesh, V.; Ramnath, N.; Ramamurthya, V. J. Photochem. 1982,
     18, 293.
19. Chattopadhyay, S.K.; Kumar, C.V.; Das, P.K. J. Photochem.
     1984, 18, 293.
20. Chattopadhyay, S.K.; Kumar, C.V.; Das, P.K. J. Photochem.
     1985, 30, 81.
21. Singh, A.; McIntyre, N.R.; Koroll, G.W. Photochem. Photobiol.
     1978, 28, 595.
22. Lindig, B.A.; Rodgers, M.A.J.; Schaap, A.P. J. Am. Chem. Soc.
     1980, 102, 5590.
23. Gandin, E.: Lion, Y.; Van de vorst, A. Photochem. Photobiol.
     1983, 37, 271.
24. Lamberts, J.J.M.; Neckers, D.C.; Tetrahedron, 1985, 41, 2183.

25. Paczkowski, J.; Neckers, D.C.; Macromolecules, 1985, 18, 1245.
26. Verlhac, J.B.; Gandemer, A.; Kraljic, I. Nouv. J. Chim. 1984, 8, 401.
27. Young, R.H.; Brewer, D.R. In Singlet Oxygen; Ranby, B.; Rabek, J.F., Eds.; Wiley: New York, 1978, p. 36.
28. Krasnovski, A.A. Photochem. Photobiol. 1979, 29, 29.
29. Byteva, I.M.; Gurinovitch, G.P. J. Lumin. 1979, 21, 17.
30. Salakhiddinov, K.I.; Byteva, I.M.; Dzhagarev, B.M. Opt. Spectrosk. 1979, 47, 881.
31. Khan, A.U.; Kasha, M. Proc. Natl. Acad. Sci. U.S.A. 1979, 76, 6047.
32. Hurst, J.R.; McDonald, J.D.; Schuster, G.B. J. Am. Chem. Soc. 1982, 104, 2065.
33. Parker, J.G.; Stanbro, W.D. J. Am. Chem. Soc. 1982, 104
34. Ogilby, P.R.; Foote, C.S. J. Am. Chem. Soc. 1982, 104, 2069.
35. Rodgers, M.A.J.; Snowden, P.T. J. Am. Chem. Soc. 1982, 104,
36. Fuke, K.; Ueda, M.; Itoh, M. Chem. Phys. Lett. 1980, 76, 372.
37. Rossbroich, G.; Garcia, N.A.; Braslavsky, S.E. J. Photochem. 1985, 31,
38. Khan, A.U. This Volume; Chapter XX.
39. Khan, A.U. Chem. Phys. Lett. 1980, 72, 112.
40. Kanofsky, J.R. J. Biol. Chem. 1983, 258, 5991.
41. Rodgers, M.A.J. J. Am. Chem. Soc. 1983, 105, 6201.
42. Knox, C.N.; Land, E.J.; Truscott, T.G. Photochem. Photobiol. 1986, 43, 359.
43. Reddi, E.; Jori, G; Rodgers, M.A.J.; Spikes, J.D. Photochem. Photobiol. 1983, 38, 639.
44. Keene, J.P.; Kessel, D.; Land, E.J.; Redmond, R.W.; Truscott, T.G. Photochem. Photobiol. 1986, 43, 117.
45. Gorman, A.A.; Rodgers, M.A.J. J. Am. Chem. Soc. 1986, 108, 5074.
46. Gorman, A.A.; Hamblett, I.; Rodgers, M.A.J. J. Photochem. 1984, 25, 115.
47. Gorman, A.A.; Rodgers, M.A.J. Chem. Phys. Lett. 1984, 120, 58.

Chapter 6

Biochemistry of Photodynamic Action

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Cells and organisms are injured and killed by exposure to light in the presence of many photo-sensitizers. This phenomenon, which typically requires molecular oxygen, is termed photodynamic action and results from the sensitized photodegrad-ation of essential biomolecules in cells. The major cellular targets are unsaturated lipids, proteins and nucleic acids. Photodynamic effects are mediated by photochemically generated singlet oxygen, free radicals, hydrogen peroxide and superoxide, depending on the sensitizer, the chemical nature of the molecule being photo-degraded, and the reaction conditions. This paper reviews the photochemical and biochemical pathways involved in the photodynamic degradation of the major classes of susceptible biomolecules.

Organisms are very sensitive to shorter wavelength ultraviolet radiation that is absorbed efficiently by essential biomolecules such as nucleic acids and proteins. Radiation in the long wavelength ultraviolet-visible range is much less harmful, in part because relatively few types of biomolecules absorb appreciably in this region of the spectrum. However, in the presence of appropriate photosensitizers, all kinds of organisms, plant and animal, single celled and multicellular, are injured and killed by light in this range (1,2). The harmful effects result from the sensitized photoalteration of critical types of biomolecules; this in turn interferes with metabolic processes and the proper function of cellular structures and organelles (cell membranes, mitochondria, nuclei, etc.).

This chapter reviews the biochemical changes that occur in different kinds of molecules of biological importance as a result of illumination in the presence of photosensitizers. Probably the first biochemical study of a photodynamic reaction was that of Professor Hermann von Tappeiner and his students who showed in 1903

that eosin sensitizes the photoinactivation of the enzymes diastase, invertase and papain under aerobic conditions (3). Von Tappeiner coined the term "photodynamic" inactivation for this phenomenon (4). Much later the inactivation was shown to result from the sensitized photooxidation of certain amino acid residues in the proteins (5). Blum (4), in his seminal monograph "Photodynamic Action and Diseases Caused by Light", suggested that the term "photodynamic action" be confined to those photosensitized reactions that require molecular oxygen and in which oxygen is consumed. Not all investigators accept this definition, but for convenience, we will use it in this review. Actually, most photosensitized reactions involving biomolecules do require molecular oxygen. There are notable exceptions, however, which cannot be covered in this review (1).

Comments on Photodynamic Mechanisms and Photosensitizers

Two principle mechanisms are involved in photodynamic reactions, termed Type I and Type II processes, respectively (6–9). In Type I reactions the light-excited sensitizer (typically in its triplet state) reacts directly with the substrate via an electron or hydrogen atom transfer with the production of free radical forms of the two reactants. These species can react further in a number of ways; in the presence of oxygen the products are often an oxidized form of the substrate and the regenerated ground state of the sensitizer. In some reactions of this type, superoxide or hydrogen peroxide is produced which can oxidize some biomolecules. Sensitizer triplet in Type II reactions interacts by energy transfer with ground state oxygen (which is a triplet) yielding ground state sensitizer and singlet oxygen; this latter species is highly electrophilic and can react with many types of biomolecules much more rapidly than does ground state oxygen (6–9).

   Although photodynamic reactions involving biomolecules sometimes appear to be simple, they more often turn out to be rather complex. Several, often competing, reaction pathways can be involved, depending on the substrate (compound being photooxidized), the sensitizer, the solvent, and the reaction conditions (reactant concentrations, pH, solvent, etc.). In many cases the primary product is unstable and decays very rapidly to secondary products. Also, the primary or subsequent products may themselves be susceptible to further photodynamic degradation (6).

   Several hundred compounds have been examined for their ability to sensitize photoreactions of biomolecules. Effective photosensitizers for biological systems include natural products such as iron-free porphyrins (coproporphyrin, protoporphyrin, uroporphyrin), flavins such as riboflavin and FMN, chlorophyll and a number of other compounds found in plants including alkaloids, extended quinones, furanocoumarins, polyacetylenes, thiophenes, etc. A large number of synthetic compounds including acridines such as acridine orange, anthraquinones, azine dyes, many ketones, a large number of synthetic porphyrins, phthalocyanines, thiazine dyes such as methylene blue, xanthene dyes such as eosin and rose bengal, etc. (2,10).

Biochemistry of Photodynamic Reactions in Solution

Biomolecules susceptible to photodynamic action include certain
organic acids, alcohols, amines, carbohydrates, nitrogen
heterocyclics, nucleic acids and certain nucleic acid bases, some
plant hormones, proteins and certain amino acids, pyrroles,
steroids, unsaturated lipids, some vitamins, etc. A very large
amount of research has been done on the effects of photodynamic
treatment in vitro (in solution) on these types of biomolecules, as
reviewed briefly in the following sections. There are several
recent short reviews of photodynamic effects on biomolecules
(1,2,7,8) and one detailed review (5); these may be consulted for a
more in-depth introduction to the literature of this area.

Photodynamic Effects on Alcohols and Carbohydrates. Alcohols and
carbohydrates, including simple sugars, polysaccharides such as
cellulose, and complex carbohydrates such as alginates, heparin and
hyaluronic acid are photooxidized slowly; anthraquinone and ketone
sensitizers are the most effective (5,6,11). These reactions
typically proceed by a Type I process. For example, a hydrogen atom
is abstracted from the alpha-carbon of alcohols; the resulting
alcohol radical reacts with oxygen, giving an aldehyde or carboxylic
acid for primary alcohols and a ketone for secondary alcohols (6).
Hexitols are photooxidized first to the hexose and then to the
corresponding hexonic acid. Cellulose is photooxidized by similar
processes giving damaged and weakened fibers ("phototendering") (6).
    Hyaluronic acid, a high molecular weight glycosaminoglycan, is a
major component of the jelly-like ground substance of animal tissues
and of the vitreous humor of the eye. The viscosity of hyaluronic
acid solutions progressively decreases on illumination in the
presence of photodynamic sensitizers; this has generally been
regarded as resulting from a free radical initiated scission of the
hyaluronic acid chain. Methylene blue sensitizes a viscosity
decrease in a reaction apparently mediated by singlet oxygen; the
change in viscosity appears to result from alterations in the
tertiary structure of the hyaluronic acid followed by only minor
depolymerization (11). Clearly much remains to be learned about the
mechanisms of photodynamic effects on complex carbohydrates.

Photodynamic Effects on Lipids. The lipids are a diverse group of
compounds including fatty acids, fats (triglycerides of fatty
acids), phospholipids, steroids and their derivatives, etc.
Unsaturated fatty acids, and fats and phospholipids containing
unsaturated fatty acids, are susceptible to photodynamic action by
both Type I and Type II processes (5). Allylic hydroperoxides are
formed initially in both mechanisms; however, the kinetics of
photooxidation and the distribution of product isomers are different
in the two cases. The Type I process is more complex, giving rise
to allylic free radicals that can react to give different
hydroperoxides as well as alcohols, epoxides and ketones. The
reaction with singlet oxygen is often much simpler, with fewer
products being formed. Polyunsaturated fats and phospholipids
appear to be photooxidized largely by a Type II reaction giving
mono- and dihydroperoxides; these initial products can undergo dark

autoxidation with further degradation and the formation of more complex mixtures of reaction products (5-7,12). The initial step in the Type II process is an "ene" reaction (13).

Cholesterol gives a characteristically different pattern of products depending on whether it is photooxidized by a Type I or a Type II process. Again, the singlet oxygen pathway gives a simpler product pattern, i.e., almost entirely the 5-alpha-hydroperoxide with only small amounts of other hydroperoxides. In contrast, in the free radical process, the 7-alpha- and 7-beta-hydroperoxides are formed along with a number of other products (5-7,14). Other steroids can also be photooxidized, including prednisolone, deoxycorticosterone and substituted hydrocortisones. For example, they are photooxidized to carboxylic acid derivatives on illumination in the presence of flavins (15). Estrone is irreversibly photobound to protein in a sensitized reaction (16), while some contraceptive steroids are decomposed by photodynamic treatment (17). These reactions may account for the photoallergy shown by some individuals using oral contraceptives.

Photodynamic Effects on Amino Acids. Of the approximately 20 amino acids that occur in proteins, only five (cysteine, a thiol; histidine, an imidazole; methionine, an organic sulfide; tryptophan, an indole; and tyrosine, a phenol) are photooxidized rapidly with photodynamic sensitizers; these amino acids all have electron-rich side chains (5,6-8). The mechanisms and kinetics of amino acid photooxidation depend on the amino acid, the sensitizer, the solvent, the pH, etc. (5). With eosin, rose bengal, proflavin and porphyrins, cysteine is photooxidized largely to cystine in a Type II process; the pH dependence of the rate indicates that the unprotonated thiol group is most reactive (5,18). The thiol free radical is produced during the hematoporphyrin-sensitized photooxidation of cysteine (19). In contrast, cysteine is photooxidized to cysteic acid with crystal violet in what appears to be a Type I reaction (20).

Histidine is rapidly photooxidized with many sensitizers. The reaction rate increases with pH in a fashion demonstrating that the unprotonated imidazole ring of histidine is the reactive site (5). Histidine and related imidazoles appear to be photooxidized by a Type II process with the formation of endoperoxides; these are very unstable, and break down rapidly with destruction of the imidazole ring and the formation of a number of secondary products (7,21). With some sensitizers, such as rose bengal, methionine is photooxidized directly to methionine sulfoxide by a Type II process at low pH, or if the amino group is blocked. At high pH with the amino group free, the primary product is dehydromethionine, which slowly hydrolyzes with the formation of methionine sulfoxide in a rather complex reaction (18,22). In contrast, with flavin and benzophenone type sensitizers, methionine is deaminated and decarboxylated by a Type I reaction to give the aldehyde, methional (23). This can be further photodegraded to yield a variety of products.

Tryptophan gives complex mixtures of products on photooxidation in reactions that depend on the sensitizer and the reaction conditions; some of the products can be further photooxidized. Both

Type I and Type II processes can apparently be involved. One
product produced by reaction with singlet oxygen is N-
formylkynurenine, which itself is a good photodynamic sensitizer
(5,24,25). Tyrosine appears to be photooxidized by both Type I and
Type II reactions; the reaction rate increases with pH in a manner
showing that the phenolate anion is the most easily photooxidized
form of the molecule. Relatively little is known of the
photooxidation products, although rupture of the tyrosine ring does
occur (5,6). Arginine and lysine have been reported to be
photooxidized under some conditions (5), and the photooxidation of
phenylalanine is sensitized by flavines (26,27). Although primary
amine groups are apparently not photooxidized, the concentration of
free amino groups in a solution of some amino acids decreases on
photooxidation; this may result from intra- or inter-molecular dark
reactions between initial oxygenation products and primary amino
groups (28).

Photodynamic Effects on Proteins. With the exception of horseradish
peroxidase and superoxide dismutase, all of the over 100 proteins
that have been examined are susceptible to photodynamic treatment
(5). Proteins examined include enzymes of all categories, blood
plasma proteins (albumins, ceruloplasmin, complement, fibrinogen,
hemopexin, hemocyanin, immunoglobulins, etc.), hormones (insulin,
glucagon, etc.), and miscellaneous proteins such as bacterial
toxins, collagen, cytochromes, eye lens crystallins, globins,
ovalbumin, ovotransferrin, snake venom proteins, spectrin, tubulin,
etc. (a detailed listing is given in ref. 5). Oxygen is consumed in
these reactions; however, little is known of its ultimate fate in
most cases. The site(s) of damage on the protein molecule is
typically one or more of the cysteinyl, histidyl, methionyl,
tryptophyl and tyrosyl residues (5). Susceptible residues located
at the surface of protein molecules will tend to be photooxidized
faster than residues buried in the interior of the protein. If the
protein is completely unfolded, all of the susceptible residues can
be photooxidized (29). In most cases peptide bonds are not broken
as a result of photodynamic treatment (5).
     Some selectivity of residue photooxidation can be obtained using
sensitizers that bind to specific sites on protein molecules
(5,30). A few proteins contain natural photosensitizing
chromophores bound in particular regions (5). For example, in the
case of the enzyme 6-phosphogluconate dehydrogenase, the enzyme
cofactor, pyridoxal, sensitizes the photodynamic modification of a
histidine residue located near the pyridoxal binding site of the
enzyme (31).
     A number of types of physicochemical changes are observed in
photodynamically-treated proteins including alterations of
absorption spectrum, aggregation properties, cofactor- and metal-
binding properties, conformation, mechanical properties, optical
rotation, solubility, viscosity, etc. (5). Increased sensitivity to
denaturation by heat or urea, and increased susceptibility to
digestion by proteases are often observed as a result of the
photodynamic treatment of proteins (32). The nature and extent of
all of these physicochemical changes depends on the protein, the
sensitizer, the reaction conditions, the degree of photooxidation of

the protein, etc. (5). Illumination of some sensitizer-protein
combinations results in the formation of covalent sensitizer-protein
photoadducts, presumably by Type I processes (5,33). In some cases,
proteins are cross-linked by photodynamic treatment giving protein
dimers, trimers, etc. The mechanisms of cross-linking are not fully
understood. In the case of spectrin, a membrane protein from
erythrocytes, it has been suggested that cross-linking results from
the interaction of a photooxidized histidyl residue on one spectrin
molecule with a free amino group on another spectrin molecule
(34). The photosensitized covalent cross-linking of proteins to DNA
(35), and the photosensitized covalent coupling of proteins to small
molecules such as tryptophan (36) also occur.
      Photodynamic treatment usually alters or destroys the normal
biological function of proteins. For example, almost all enzymes
lose their biocatalytic activity as a result of the destruction of
essential amino acid residues in the active site or binding site of
the enzyme or by the alteration of residues located elsewhere that
are necessary for the normal catalytic conformation of the enzyme
(5). The antigenicity of some proteins as well as their ability to
react with antibodies directed toward them is decreased. The
biological functions of peptide hormones such as angiotensin,
glucagon, insulin, etc. are destroyed by photodynamic treatment, and
protein toxins from bacteria, plants and snakes are inactivated (5).

Photodynamic Effects on Purines and Pyrimidines. The sensitized
photooxidation of biologically important purines and pyrimidines,
their nucleosides and nucleotides, and some related compounds has
been examined using a number of different sensitizers (5,37). In
general, under physiological conditions, purines are photooxidized
more readily than pyrimidines, and guanine and its derivatives are
more susceptible than other purines (5). Ring substituents on
nucleic acid bases can have a major effect on the sensitivity to
photooxidation. For example, with hematoporphyrin, 5-aminouracil
and 5-hydroxyuracil are photooxidized very much faster than 5-
methyluracil (38). The photooxidation of guanine and some of the
other nucleic acid bases can occur by both Type I and Type II
processes, with the relative involvement of each pathway depending
on the photosensitizer used. Rose bengal sensitizes the
photooxidation of 3',5'-di-0-acetyl-2'-deoxyguanosine largely by a
Type II mechanism; in contrast, riboflavin and benzophenone
sensitize the reaction primarily by a Type I process (39).
Riboflavin also sensitizes the photooxidation of 2'-deoxyguanosine
by a Type I process (40). With many sensitizers, the rate of
photooxidation of guanine and its derivatives increases with
increasing pH in a manner indicating that the anionic forms of these
compounds, as with some amino acids, are the most sensitive to
attack. The photooxidation rates of thymine and uracil also
increase with pH (5).
      Relatively little is known about the mechanistic details of the
photooxidation of purines and pyrimidines. Typically, complex
arrays of reaction products are formed that are probably derived
from unstable dioxetanes, endoperoxides or hydroperoxides (7,21).
The methylene blue sensitized photooxidation of guanosine results in
the ultimate production of guanidine, ribose, ribosylurea and urea

(41). The Type I oxidation products of 3',5'-di-0-acetyl-2'-
deoxyguanosine include two anomers of 3',5'-di-0-acetyl-2'-deoxy-
erythropentofuranose plus several unidentified compounds. The
predominant singlet oxygen oxidation products of this guanosine
derivative are N-(3,5-di-0-acetyl-2-deoxy-erythropentofuranosyl)-
cyanuric acid and 9-(3,5-di-0-acetyl-2-deoxy-erythropentofuranosyl)-
4,8-dihydro-4-hydroxy-8-oxoguanine (37). The primary photooxidation
product of uracil, as determined at very low temperatures, appears
to be a very unstable hydroperoxide (42).

Photodynamic Effects on Nucleic Acids. Nucleic acids are
photooxidized on illumination in the presence of a number of
different kinds of photosensitizers; in essentially all cases,
photodynamic treatment of nucleic acids and synthetic
polynucleotides preferentially destroys guanine residues (5,37).
For example, it has been shown that hematoporphyrin, which does not
intercalate into nucleic acids, specifically sensitizes the
photoalteration of guanine residues in single-stranded DNA;
subsequent treatment with base results in chain breaks at each
guanine residue. Methylene blue, which, like other basic dyes
intercalates into the nucleic acid helix, specifically sensitizes
the photoalteration of guanine residues in both single-stranded and
double-stranded DNA. Although the hematoporphyrin radical anion is
produced in good yield on illumination in the presence of DNA, it
does not interact with the nucleic acid. Singlet oxygen appears to
be the reactive species with both hematoporphyrin and methylene blue
(43). In contrast, photodynamic treatment of DNA with rose bengal
(44) or with riboflavin (45) generates single-strand chain breaks,
apparently by interaction of the triplet sensitizers with the
nucleic acid. Singlet oxygen does not appear to be involved.
Superoxide and hydrogen peroxide have also been suggested as being
reactants in the photodynamic degradation of nucleic acids (46).
     In addition to strand breakage, photodynamically-treated nucleic
acids show conformational alterations, spectral shifts, a decrease
in solution viscosity and melting temperature, and an increased
susceptibility to enzymatic digestion (5). Major changes in
biological activity also occur. For example, tobacco mosaic virus
RNA loses its ability to infect tobacco plants, DNA transforming
principle from bacteria is destroyed, and transfer RNA is
inactivated; further, the messenger, template and translational
activities of nucleic acids are altered (5,46,47). Mutations can be
produced in bacteriophage DNA by photodynamic treatment (48).

Photodynamic Effects on Miscellaneous Biomolecules. In addition to
the major categories described above, a number of other kinds of
biomolecules are sensitive to photodynamic attack (1). For example,
ascorbic acid is photooxidized with porphyrin sensitizers; the
ascorbate free radical is produced in the reaction (49). The plant
hormone, indole-3-acetic acid, is photooxidized using FMN; competing
Type I and Type II processes are involved (50). Eosin Y and
methylene blue sensitize the photooxidation of alpha-ketoglutaric
acid in a singlet oxygen mediated process; succinic acid and carbon
dioxide are produced in the reaction (51). The illumination of
phytol in the presence of rose bengal or methylene blue gives two

allylic hydroperoxides in a reaction involving singlet oxygen
(52). Squalene is photooxidized with chlorpromazine via a singlet
oxygen reaction with the formation of peroxidized products (53).
The flavanol, quercetin, is slowly photooxidized in a self-
sensitized process, as well as in a reaction sensitized by
riboflavin. The rate of the flavine-sensitized photooxidation is
increased 10-fold in the presence of EDTA in a reaction inhibited by
superoxide dismutase, suggesting that the process is mediated, at
least in part, by superoxide (54). Vitamin E (alpha-tocopherol)
reacts with singlet oxygen produced by photodynamic sensitizers by a
rapid physical quenching process, and by a slower, irreversible
reaction that gives two isomeric hydroperoxydienones (55). The
illumination of vitamin E in the presence of hematoporphyrin
derivative leads to the uptake of oxygen and the formation of the
vitamin E chromanoxyl free radical (56). Photodynamic treatment of
vitamin B12 with methylene blue as sensitizer results in two
photooxygenated products (57). Illumination of natural pheomelanin
pigments and model melanins results in a slow uptake of oxygen; the
rate is greatly enhanced by added rose bengal in a reaction that
appears to involve singlet oxygen, at least in part. Rose bengal
also sensitizes an increase in the photoproduction of free radicals
in melanins (58).

Biochemistry of Photodynamic Reactions in Cells and Organelles

Information obtained on photosensitized reactions in solution
studies cannot always be applied directly to studies with cells.
This is because cells and subcellular structures are non-
homogeneous, with regions that differ widely in chemical makeup.
Thus cells provide a large range of microenvironments with different
physicochemical properties. As a result, the photosensitizing
properties of sensitizers can depend on their particular cellular
environment; similarly the reactions of substrates may be different
in different regions of the cell.
    Most types of biomolecules in intact cells and in isolated
cellular organelles show the same kinds of biochemical changes on
photodynamic treatment as observed in solution (1,2). For example,
susceptible amino acid residues in cell membrane proteins are
photooxidized efficiently (1,59). This results in the photodynamic
inactivation of a number of membrane associated enzymes including
glyceraldehyde-3-phosphate dehydrogenase, ATPases, acetylcholin-
esterase, etc. Enzymes associated with mitochondria, such as
succinic dehydrogenase, ATPase, and adenylate kinase are also
inactivated efficiently with some sensitizers (60); among other
effects, this may result in decreased levels of ATP in treated
cells, which can interfere with a number of different cellular
activities (61). Soluble enzymes in the cytosol of the cell are
also sensitive. Illumination of photosensitized cell membranes,
e.g., red blood cell ghosts, in the presence of porphyrins, gives a
singlet oxygen—mediatd peroxidation of the membrane lipids; the rate
of peroxidation is significantly increased by low concentrations of
ascorbate (62). The photodynamic treatment of isolated microsomes
results in the peroxidation of the component lipids and in the
inactivation of the mixed function oxidase system. Illumination of

hepatic microsomes from rats pretreated with hematoporphyrin
derivative results in a rapid destruction of cytochrome P-450
(63). As in solution, sensitized photocross-linking reactions occur
with biomolecules in cell organelles and in intact cells. Actually,
such reactions may be favored in cells as compared to solution
systems because of the highly ordered structure of cells (64). DNA
strand breaks occur in murine fibroblast cells illuminated in the
presence of hematoporphyrin derivative; this apparently results from
the photooxidation of guanine residues in the nucleic acid (65).

Summary

We now understand the initial reactions involved in the biochemistry
of photodynamic action reasonably well, i.e., the production and
properties of the excited states of sensitizers and the interaction
of triplet sensitizers with ground state oxygen to give singlet
oxygen. The interaction of singlet oxygen with biomolecules is
becoming clearer, but the reactions of triplet sensitizers with
biological substrates via free radical processes are more complex.
Much remains to be learned about the mechanistic organic chemistry
of these reactions. Finally, although progress is being made, our
understanding of photosensitized reactions at the cellular and
organismal levels is still very incomplete. There is much work yet
to be done on the biochemistry of photodynamic action.

Literature Cited

1.   Spikes, J.D. In Photoimmunology; Parrish, J.A.; Kripke, M.L.;
     Morison, W.L., Eds.; Plenum: New York, 1983; Chapter 2.
2.   Spikes, J.D. In The Science of Photomedicine; Regan, J.D.;
     Parrish, J.A., Eds.; Plenum: New York, 1982; Chapter 5.
3.   Tappeiner, H. v. Ber. Deut. Chem. Ges. 1903, 36, 3035.
4.   Blum, H.F. Photodynamic Action and Diseases Caused by Light;
     Hafner Publishing Company: New York, 1964.
5.   Straight, R.C.; Spikes, J.D. In Singlet O_2; Frimer, A.A., Ed.;
     CRC Press: Boca Raton, 1985; Vol IV, Chapter 2.
6.   Foote, C.S. In Free Radicals in Biology; Pryor, W.A., Ed.;
     Academic: New York, 1976; Vol II, Chapter 3.
7.   Foote, C.S. In Oxygen and Oxy-Radicals in Chemistry and
     Biology; Rodgers, M.A.J.; Powers, E.L., Eds.; Academic: New
     York, 1981; p 425.
8.   Foote, C.S. In Porphyrin Localization and Treatment of Tumors;
     Doiron, D.R.; Gomer, C.J., Eds.; Alan R. Liss: New York, 1984;
     p 3.
9.   Laustriat, G. Biochimie 1986, 68, 771.
10. Towers, G.H.N. Prog. Phytochem. 1980, 6, 1983.
11. Andley, U.P.; Chakrabarti, B. Biochem. Biophys. Res. Commun.
     1983, 115, 894.
12. Terao, J.; Matsushita, S. Agric. Biol. Chem. 1981, 45, 601.

13. Frimer, A.A.; Stephenson, L.M. In Singlet O$_2$; Frimer, A.A., Ed.; CRC Press: Boca Raton, 1985; Vol II, Chapter 3.
14. Teng, J.I.; Smith, L.L. J. Am. Chem. Soc. 1973, 95, 4060.
15. Jasiczak, J.; Smoczkiewica, M.A. Tetrahedron Lett. 1985, 26, 5221.
16. Sedee, A.G.J.; Beijersbergen van Henegouwen, G.M.J.; Lusthof, K.J.; Lodder, G. Biochem. Biophys. Res. Commun. 1984, 125, 675.
17. Sedee, A.; Vanhenegouwen, G.B. Arch. Pharm. 1985, 318, 111.
18. Ando, W.; Takata, T. In Singlet O$_2$; Frimer, A.A., Ed.; CRC Press: Boca Raton, 1985; Vol III, Chapter 1.
19. Buettner, G.R. FEBS Lett. 1985, 177, 295.
20. Gennari, G.; Cauzzo, G.; Jori, G. Photochem. Photobiol. 1974, 20, 497.
21. Wasserman, H.W.; Lipshutz, B.H. In Singlet Oxygen; Wasserman, H.W.; Murray, R.W., Eds.; Academic Press: New York, 1979; Chapter 9.
22. Sysak, P.K.; Foote, C.S.; Ching, T-Y. Photochem. Photobiol. 1977, 26, 19.
23. Bowen, J.R.; Yang, S.F. Photochem. Photobiol. 2975, 21, 201.
24. George, M.V.; Bhat, V. Chem. Rev. 1979, 79, 447.
25. Nakagawa, M.; Yokoyama, Y.; Kato, S.; Hino, T. Tetrahedron 1985, 41, 2125.
26. Ishimitsu, S.; Fujimoto, S.; Ohara, A. Chem. Pharm. Bull. 1985, 33, 1552.
27. Rizzuto, F.R.; Spikes, J.D.; Coker, G.D. Photobiochem. Photobiophys. 1986, 10, 149.
28. Straight, R.C.; Spikes, J.D. Photochem. Photobiol. 1978, 27, 565.
29. Jori, G.; Galiazzo, G.; Tamburro, A.M; Scoffone, E. J. Biol. Chem. 1970, 245, 3375.
30. Jori, G. An. Acad. Bras. Cien. 1973. 45, 33.
31. Rippa, M.; Pontremoli, S. Arch. Biochem. Biophys. 1969, 103, 112.
32. Hopkins, T.R.; Spikes, J.D. Photochem. Photobiol. 1970, 12, 175.
33. Brandt, J. Methods Enzymol. 1977, 46, 561.
34. Verweij, H.; Dubbleman, T.M.A.R.; Van Steveninck, J. Biochim. Biophys. Acta. 1981, 647, 87.
35. Helene, C. In Aging, Carcinogenesis and Radiation Biology; Smith, K.C., Ed.; Plenum Press: New York, 1976; p 149.
36. Hemmendorf, B.; Brandt, J.; Anderson, L.-O. Biochim. Biophys. Acta. 1981, 667, 15.
37. Cadet, J.; Berger, M.; Decarroz, C.; Wagner, J.R.; Van Lier, J.E.; Ginot, Y.M.; Vigny, P. Biochemie 1986, 68, 813.
38. Jori, G.; Spikes, J.D. In Topics in Photomedicine; Smith, K.C., Ed.; Plenum Press: New York, 1984; p 183.
39. Cadet, J.; Decarroz, C.; Voituriez, L.; Gaboriau, F.; Vigny, P. In Oxygen Radicals in Chemistry and Biology; Bors, W.; Saran, M.; Tait, D., eds.; Walter de Gruyter: Berlin, 1984; p 485.
40. Ennever, J.F.; Speck, W.T. Pediatr. Res. 1981, 15, 956.
41. Matsuura, T.; Saito, I. General Heterocyclic Chem. 1976, 4, 456.

42. Vickers, R.S.; Foote, S. Boll. Chim. Farm. 1970, 109, 599.
43. Kawanishi, S.; Inoue, S.; Sano, S.; Aiba, H. J. Biol. Chem.
     1986, 261, 6090.
44. Peak, M.J.; Peak, J.G.; Foote, C.S.; Krinsky, N.I. J.
     Photochem. 1984, 25, 309.
45. Korycka-Dahl, M.; Richardson, T. Biochim. Biophys. Acta. 1980,
     610, 229.
46. Amagasa, J. Photochem. Photobiol. 1981, 33, 947.
47. Spikes, J.D.; MacKnight, M.L. In Photochemistry of
     Macromolecules; Reinisch, R.F., Ed.; Plenum Press: New York,
     1970; p 67.
48. Piette, J.; Calberg-Bacq, D.M.; Van de Vorst, A. Mol. Gen.
     Genet. 1978, 167, 95.
49. Buettner, G.R.; Need, M.J. Cancer Lett. 1985, 25, 297.
50. Miyoshi, N.; Fukuda, M.; Tomita, G. Photobiochem.
     Photobiophys. 1986, 11, 57.
51. Gandhi, P.; Dubey, R.; Bokadia, M.M.; Sharma, T.C. Curr. Sci.
     1985, 54, 37.
52. Mihara, S.; Tateba, H. J. Org. Chem. 1986, 51, 1142.
53. Fujita, H.; Matsuo, I.; Okazaki, M.; Yuoshino, K.; Ohkido, M.
     Dermatol. Res. 1986, 278, 224.
54. Takahama, U. Photochem. Photobiol. 1985, 42, 89.
55. Clough, R.L.; Yee, B.G.; Foote, C.S. J. Am. Chem. Soc. 1979,
     101, 683.
56. Buettner, G.R. In Primary Photo-Processes in Biology and
     Medicine; Bensasson, R.V.; Jori, G.; Land, E.J.; Truscott,
     T.G., Eds.; Plenum: New York, 1985; p 341.
57. Kraeutler, B.; Stepanek, R. Agnew. Chem. 1985, 97, 71.
58. Sarna, T.; Menon, I.A.; Sealy, R.C. Photochem. Photobiol.
     1985, 42, 529.
59. Moan, J.; Vistnes, A.I. Photochem. Photobiol. 1986, 44, 15.
60. Fu, N.; Yeh, S.; Chang, C.; Zhao, X.; Chang, L. Adv. Exp. Med.
     Biol. 1985, 193, 161.
61. Hilf, R.; Murant, R.S.; Narayanan, U.; Gibson, S.L. Cancer
     Res. 1986, 46, 211.
62. Girotti, A.W.: Thomas, J.P.; Jordan, J.E. Photochem.
     Photobiol. 1985, 41, 267.
63. Das, M.; Dixit, R.; Mukhtar, H.; Bickers, D.R. Cancer Res.
     1985, 45, 608.
64. Jori, G.; Spikes, J.D. In Oxygen and Oxy-Radicals in Chemistry
     and Biology; Rodgers, M.A.J.; Powers, E.L., Eds.; Academic
     Press: New York, 1981; p 441.
65. Dubbelman, T.M.A.R.; Boegheim, J.P.J.; Van Steveninck, J. In
     Primary Photo-Processes in Biology and Medicine; Bensasson,
     R.V.; Jori, G.; Land, E.J.; Truscott, T.G., Eds.; Plenum: New
     York, 1985; p 397.

Chapter 7

Photodynamic Modification of Excitable Cell Function

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Photodynamic treatment of electrically excitable cells
from nerve and muscle causes a dose-dependent
perturbation in their signaling function. Nerve cell
axons, particularly at neuromuscular junctions, may be
induced to fire action potentials. Voltage clamp
analyses of photomodified lobster axons reveal a block
of sodium channels and an inhibition of inactivation
gating in unblocked channels. Potassium channels
(delayed rectifiers) are less susceptible, but also
become blocked and have a slowed activation. An
unidentified ion leakage is created that is probably
responsible for triggering the light-induced firing.
The susceptibility to firing observed in vertebrate
neuromuscular junctions suggests that insect
neuromuscular junctions may be a likely target for
light-activated pesticides.

The nervous system has been implicated as a key target of light-
activated pesticides suggested in part by behavioral abnormalities
indicative of loss of neuromuscular control (1). Although there is
no direct evidence to support this contention, it is known that the
nervous systems of all organisms are highly susceptible to
perturbation by toxins and pharmacological agents (2), and nerve
cells from non-insect species have been shown to be easily
photomodified (3-9). The purpose of this chapter is to describe
existing studies on photodynamic modification of excitable cells and
point out how these findings might apply to the actions of light-
activated pesticides.
     Nervous systems carry out the function of signaling, from one
region of a cell to another and from one end of an organism to the
other. All nervous systems are composed of similar elements and
behave according to uniform principles at the cellular level, in
much the same way that complex electronic devices of diverse
function are composed of identical elementary components (10).
Students of neurobiology have usually chosen species for study more

for convenience, such as ready availability and ease of dissection, than for any uniqueness in principle of operation. Although studies on the photodynamic modifications of nervous systems have been carried out on organisms other than those that are targets for light-activated pesticides, one may cautiously extrapolate across species and generalize results from one species to all others. Thus it is very likely that mechanisms studied on traditional preparations such as squid nerves and frog muscles apply equally to insect excitable cells.

The common elemental processes found in all nervous systems include conduction of nerve impulses; the stimulation or inhibition of one cell by another at synaptic contiguities; and spontaneous excitation in appropriate pacemaker and sensory transducer cells. Many of these same events occur in muscle tissue as well. Disturbance in any of these processes by photodynamic means is potentially lethal.

Historical Background

Nerve-muscle preparations. The first substantial investigation of photodynamic modification of excitable cells was a series of studies by Lippay initiated in the late 1920s on nerve-muscle preparations from frogs, i.e., isolated frog skeletal muscles with the distal portions of the motor nerves that innervate them still attached (11-13). He found that illumination of muscle sensitized with any of several xanthene sensitizers or hematoporphyrin led to contractions. Some contractions were slow and sustained; others were rapid and spasm-like. Lippay proposed that the spasms were triggered by light-induced firing in the motoneurons. Research in subsequent years has amply confirmed Lippay's hypothesis (14-17). Recordings in motoneurons show that action potentials originate in the distal portions of nerve near the muscle and travel antidromically away from the muscle. They also activate the neuromuscular synapse, triggering contraction. If reversible neuromuscular blocking agents are added, the light-induced contractile spasms can be blocked reversibly.

In vertebrates neuromuscular transmission is accomplished by release of acetylcholine, stored in small vesicles within the presynaptic nerve terminal. A presynaptic action potential causes the emptying of the contents of many such vesicles into the narrow space between the nerve membrane and muscle membrane, the acetylcholine then acting to depolarize the muscle membrane. Normally the acetylcholine is rapidly degraded immediately following the nerve impulse by acetylcholinesterase present in high concentration in the cleft area. An additional finding on sensitized nerve-muscle preparations from frogs is a light-induced decline in the acetylcholinesterase activity (15). With acetylcholine hydrolyzed more slowly, a lingering depolarizing action on the muscle cell occurs. Single nerve action potentials, that normally trigger single muscle action potentials and subsequent contractile twitches, now trigger multiple action potentials and twitches. Thus the stimulating action of nerve on muscle for both light-induced and electrically stimulated firing is amplified. In insects the neuromuscular transmitter is L-glutamate, and its

inactivation is mainly by uptake into glial cells rather than by enzymatic degradation (18). Whether insect neuromuscular junctions are as sensitive to photodynamic perturbation as those in frogs is unknown.

A direct photodynamic action on muscle membrane, not involving neuromuscular transmission, has also been described (19). The muscle slowly depolarizes and may break into spontaneous firing, thereby triggering contraction without any nerve activity. The muscle action potentials also become increased in duration. A second direct action on muscle is the slow contracture originally described by Lippay, and confirmed by others (14). It occurs even with neuromuscular transmission blocked and the muscle made inexcitable by depolarization with elevated extracellular potassium. The basis for the slow light-induced contracture is unresolved at present.

These photodynamic studies on nerve-muscle preparations were carried out over a span of many decades, without benefit of recent technical advances or of insights into physiological mechanism revealed by these techniques. While the findings of all investigators are not in total harmony, most agree that the most sensitive target is some region of the nerve terminal. The light doses required to cause major direct muscle effects are larger than those needed to evoke light-induced firing in the motoneurons. All of the reported light-induced effects are photodynamic--i.e., require oxygen--and seem to be independent of the particular sensitizer used. Minor conflicts between results in different studies may reflect differences in the distribution of sensitizer within the complex anatomy of the muscle at the time of illumination.

Giant axons. Almost all investigations on photodynamic modification of the nervous system have been carried out on single giant axons isolated from marine animals such as squid, cuttlefish, and lobster. Most of the work prior to 1970 was performed by Chalazonitis and coworkers in France and by Lyudkovskaya and coworkers in the USSR (3-8). The axon region of a nerve cell is relatively "simple" in that its signaling function is limited to conducting impulses, initiated at one end or in other cell regions, along its length to synaptic terminals at the other end. However, despite a stereotyped normal function, axons are capable of rather complex behavior. The electrophysiology of axons can presently be studied at several levels of sophistication, from simple extracellular recording for detection of action currents associated with the passage of nerve impulses, to "patch clamp" methods for observing the behavior of individual macromolecular ion channels. The studies of Chalazonitis, Lyudkovskaya, and a few others who preceded them many decades ago employed either extracellular recording or simple intracellular recording with a pipet electrode inserted into the axon. These methods permit detection of firing patterns and subthreshold membrane depolarization, but do not allow one to describe light-induced changes in membrane permeability. Use of the voltage clamp method, to be discussed further on, has revealed how ionic permeabilities are photodynamically changed.

Chalazonitis and Lyudkovskaya both demonstrated that
sensitizer-treated giant axons would commence repetitive firing
during illumination, preceded by a latent period during which a slow
depolarization occurred. The firing continued after cessation of
illumination for varying periods of time, but then stopped.
Sometimes the membrane potential would jump to a steady depolarized
level during illumination for several seconds and then return to a
value near the normal resting potential. In a separate study of
photodynamic modification of giant axons, Lyudkovskaya described
rather different results (8). In this case no light-induced firing
was reported. Instead Lyudkovskaya found major perturbations in the
shape of electrically stimulated action potentials. They developed
a long plateau between the rising and falling phases, and the
duration increased from a typical 1 or 2 ms up to hundreds of
milliseconds. Both Chalazonitis and Lyudkovskaya described the
light-induced firing as reversible, while the prolongation of
electrically stimulated action potentials was said to be
irreversible.

In 1968 Pooler initiated an investigation of photodynamic
modification of giant axons from lobsters (20). He found an
irreversible prolongation of electrically stimulated action
potentials, as did Lyudkovskaya, but no light-induced firing. In a
subsequent clarifying study designed to reconcile the conflicts in
reported findings, he found that light-induced firing could be
initiated in lobster giant axons if the bathing solution was free of
calcium (21). Chalazonitis and Lyudkovskaya had used such a
condition in their studies describing light-induced firing. Nerve
cells become hyperexcitable in solutions devoid of calcium, but also
deteriorate slowly if a calcium-free condition is maintained (22).
Thus giant axons may be photodynamically induced into firing in
conditions of low-calcium hyperexcitability. The firing is
apparently reversible, in that firing ceases at some point following
termination of illumination and may usually be restarted by another
dose of light. The reversibility is apparent, but not real, because
the cessation of firing is due in part to deterioration (and other
factors to be described later) rather than to removal of the
photomodification that initiates the firing.

Voltage Clamp Analysis of Membrane Channel Function

Technical background. Much of our present understanding of
excitable cell function stems from the studies of Hodgkin, Huxley
and Katz, who applied the voltage clamp technique to giant axons
from squid (23). When under voltage clamp the membrane potential in
a restricted small area of membrane is controlled by electronic
feedback. Usually the membrane potential is held at a negative
level from which a sequence of step depolarizations are applied. At
each value of potential to which the membrane is clamped, the
electrochemical driving force for ion flux is fixed. Therefore the
time course of current flow by any ion species reflects the time
course of membrane permeability to that ion, usually expressed in
terms of conductance (see Equation 1) (24). The total current
through an axon membrane may be resolved into three main components:
sodium current, potassium current, and a non-specific leakage

current. Currents are related to membrane conductance and driving forces as expressed in Equation 1, where V is the membrane potential, E is the reversal potential of the appropriate ion, and g is the conductance.

$$I = g \ (V - E) \hspace{3cm} (1)$$

The non-specific leakage conductance is considered to be constant in a normal membrane, while sodium and potassium conductances are clearly voltage- and time-dependent. Their response to a stimulus underlies the characteristic nerve impulse when the membrane potential is not controlled by electronic feedback.

Investigation subsequent to the classic studies by Hodgkin, Huxley and Katz demonstrates that the ionic conductance in a given finite region of membrane is the sum of the conductance of all the individual ion channels in that membrane. Each channel has a polypeptide containing a pore spanning the membrane that has a region of restriction, giving rise to ionic selectivity (25). Most channels also contain one or more separate "gate" regions that occlude the pore under some conditions and open to a non-occluding conformation under other conditions. In typical voltage clamp experiments the membrane region under study contains up to a million channels. Ion conductances change smoothly over time in response to a step change in potential. This smooth change represents the ensemble average of many individual channels, each being either open or closed. Thus the time course of the ensemble average reflects the time course of probability that a given channel type is open.

Sodium channels. When an axon membrane is depolarized, many sodium channels open (activate) but then close (inactivate) with a somewhat slower time course. Most investigators feel that activation is the opening of a gate that is closed when the membrane is polarized, and inactivation is the closing of a physically separate gate that is normally open when the membrane is polarized. When the membrane is repolarized there is a reversal of the events that occur during depolarization. The activation gate closes and the inactivation gate opens.

Photodynamic Modification of Lobster Giant Axons

Block of sodium channels. Photodynamic perturbation of sodium channel function can be expressed in terms of changes in gating variables and maximum conductance. Voltage clamp analysis of photomodified lobster giant axons reveals a decrease in the maximum sodium conductance. During illumination at a constant dose rate the decrease follows a simple survival curve, i.e., an exponential time course toward a zero asymptote (26). The simplest interpretation of this behavior is that increasing numbers of individual channels become totally blocked. At large doses essentially all channels are blocked and no measurable sodium current flows during a depolarization. The rate constant for the development of block during illumination varies linearly with dose rate and depends heavily on the species of sensitizer. When different sensitizers are compared under conditions of equal absorbed dose rates, the rate

of channel block becomes a useful assay to compare their potency. For example, within the fluorescein family of sensitizers rose bengal is by far the most potent (27).

Perturbation of sodium channel gating. Illumination also causes a complex disturbance of the inactivation gate in non-blocked channels, leading to a prolonged flow of sodium current in response to depolarization (26). Following any finite light dose, therefore, there are three subpopulations of sodium channels: normal, blocked, and unblocked with modified inactivation gating. The relative numbers of these subpopulations are shown schematically in Figure 1 as a function of light dose. Note that the number with perturbed inactivation gating rises with light dose up to a maximum but then falls at high doses because the majority of the channels become blocked.

Disturbance of sodium channel inactivation reveals itself in the kinetics of inactivation and its voltage dependence. At a light dose sufficient to block about 50% of the channels, the rate at which unblocked open channels inactivate during a depolarization decreases by roughly 50% (26). At the same time, the steady-state inactivation versus voltage relation is distorted. The steady-state voltage dependence for activation and inactivation gating may be expressed in terms of the gating parameters m and h of the Hodgkin-Huxley model according to Equations 2 and 3, where V is membrane potential, V_m and V_h are the membrane potentials at which the gating parameters are at half maximum, and the k's indicate the steepness of the voltage dependence:

$$m_\infty = 1/(1 + \exp((V - V_m)/k_m)) \qquad (2)$$

$$h_\infty = 1/(1 + \exp((V - V_h)/k_h)) \qquad (3)$$

Equation 3 may be revised to include a non-inactivated fraction, f, as in Equation 4:

$$h_\infty = (1 - f)/(1 + \exp((V - V_h)/k_h)) + f \qquad (4)$$

New experiments on lobster axons show the development of a foot in the inactivation curve such that some channels fail to inactivate at all at potentials near zero (see Figure 2). A similar behavior has been found on squid giant axons (28).

Possible photodynamic perturbation of activation gating is small at best and below the resolution of the measurements. The activation parameter values for photomodified axons are not significantly different from those of normal axons (Figure 2). An earlier investigation revealed no change in the kinetics of activation either (26). Thus it appears that photomodification of sodium channel gating is limited to the inactivation component.

Inactivation gating is a complex process. Inactivation occurring as the closure of open channels during a large depolarization may be different from inactivation occurring during a small depolarization that does not activate many channels (29). This second form of inactivation is called conditioned inactivation because it is measured by "conditioning" the membrane with a small
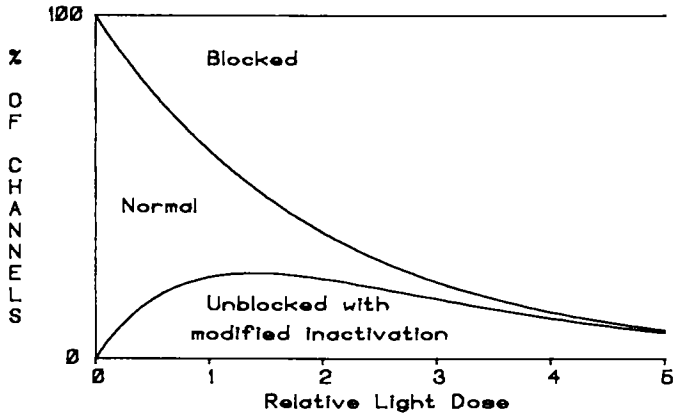
Figure 1. Schematic illustration of photodynamic modification of sodium channels showing division of total population into three fractions. Before illumination all channels are normal, while after large light doses all are blocked. The unblocked channels with modified inactivation reach a maximum at an intermediate light dose.
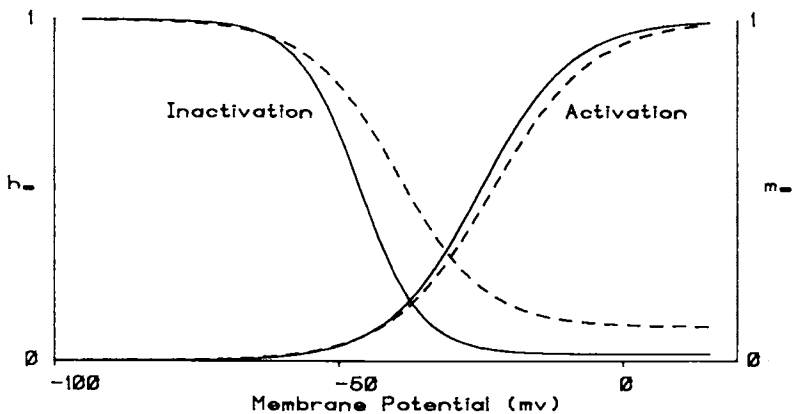
Figure 2. Steady state activation and inactivation versus membrane potential for normal axons (continuous curves) and photomodified axons (dashed curves). The curves are plots of Equations 2 and 4 using mean parameter values obtained from 10 measurements each on normal axons and photomodified axons sensitized with 5 μM acridine orange and illuminated for a time sufficient to block 50% of the sodium conductance.

depolarization and then testing for the amount of inactivation
during a subsequent large depolarization. New experiments show the
kinetics of conditioned inactivation on photomodified lobster giant
axons to be unaltered, and furthermore, the kinetics of recovery
from inactivation (the opening of the inactivation gate upon
repolarization) are not perturbed either (Figure 3). Therefore,
photodynamic modification of sodium channel gating is not only
limited to inactivation, but to that aspect observed as the closure
of open channels.

Perturbation of potassium channels and leakage. There are many
kinds of potassium channels in excitable cells. The predominant
type of potassium channel in axons is called a delayed rectifier.
Its gating has been modeled by the n parameter of the Hodgkin-Huxley
model (24). More recent study of delayed rectifiers reveals the
existence of a slow component in the kinetics not dealt with in the
Hodgkin and Huxley analysis, suggesting two populations of delayed
rectifier (30). A description of photodynamic perturbation of
delayed rectifiers is presently incomplete because of uncertainties
in the behavior of normal channels. Nevertheless, certain facts are
apparent. First, potassium channels in axons are blocked by
photodynamic treatment. The susceptibility, however, is less than
that of sodium channels. If the survival of unblocked potassium and
sodium channels is compared under identical reaction conditions, the
rate of potassium channel decay is only about 20% as great (21).
Second, the activation gating of potassium channels is disturbed
(for sodium channels it is not). The rate of activation is
decreased and the slow component becomes more prominent. It is not
clear whether this represents a selective block of the fast
component, making the overall kinetics appear slower, or whether the
activation is truly slowed. In any event the overall effect is a
decrease in the level of potassium conductance reached at a given
time following the start of a step depolarization.
     The modification that potentially has the greatest
significance for axon function--an increase in leakage--has not been
studied systematically. This is because the vast majority of
voltage clamp studies of photodynamic modification have been carried
out on lobster giant axons, on which leakage cannot be measured for
technical reasons (31). However, voltage clamp studies performed on
squid giant axons show a consistent increase in leakage
(unpublished).

Interpretation of Nerve Cell Experiments

Two of the key observations on nerve cells seem to be in conflict.
Many studies describe photodynamic modification as excitatory:
nerve cells are induced to fire during light. Yet voltage clamp
analysis shows that sodium channels become blocked by light--clearly
an inhibitory action. (Local anesthetics work by blocking sodium
channels in axons.) The resolution lies in the fact that axons have
far more than the minimum density of sodium channels required to
sustain action potentials; thus events that stimulate an axon to
fire may proceed even though sodium channels are being blocked
simultaneously. (If they all become blocked, then firing will
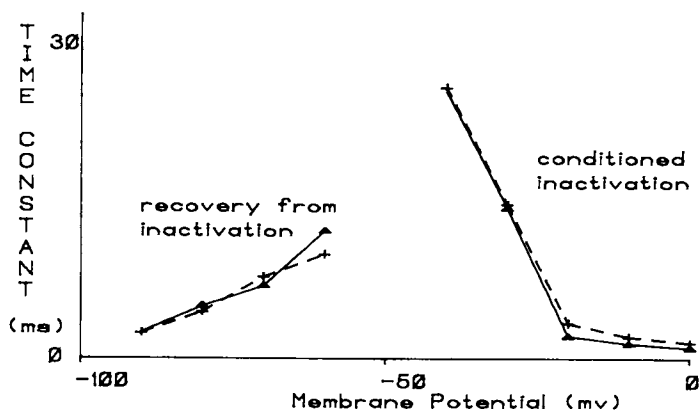
Figure 3. Time constant for conditioned inactivation and
removal of inactivation in normal axons (triangle symbols,
continuous lines) and photomodified axons (plus symbols, dashed
lines). Each point is the mean of 11 or more observations.
Reaction conditions are the same as in Figure 2. The lack of
photodynamic effect stands in contrast to the slowing of
inactivation when assessed as the closure of open channels (26).

cease.) The light-induced firing appears to be triggered by a light-induced depolarization that acts as a stimulus analogous to a sensory generator potential. The depolarization, in turn, is probably a result of the increase in leakage. However, this interpretation must be considered speculative until more detail is learned about the light-induced leakage--specifically the ion species involved. In theory, depolarization can be brought about by a decrease in permeability to an ion species with an equilibrium potential more negative than the resting potential, such as potassium. Kohli and Bryant's brief report on photodynamic depolarization of skeletal muscle cells (19) states that the effect was not seen when choline was substituted for sodium, suggesting that the depolarization is caused by an anomalous rise in sodium permeability.

Light-induced firing is easy to produce on nerve-muscle preparations, but with much more difficulty on giant axons from marine animals. Most, and possibly all, of the light-induced firing described by Lyudkovskaya and Chalazonitis was on giant axon preparations made hyperexcitable by lowering the calcium concentration in the reaction medium. Pooler described light-induced firing on lobster axons in calcium-free media, but not with high calcium concentrations present. On nerve-muscle preparations the terminal portions of the motoneurons evidently possess a high susceptibility to light-induced firing even in conditions of normal calcium. The reasons for this are presently unknown.

The reversibility of light-induced firing described by Chalazonitis and Lyudkovskaya was shown to be an apparent reversibility, due in part to a slow deterioration in calcium-free solutions. If a nerve cell is hyperexcitable and teetering on the edge of firing because of low calcium and/or a light-induced depolarization, any small influence can trigger firing or stop existing firing. Prolonged depolarization induces another form of inactivation known as slow inactivation, not ordinarily seen on the time scale of an action potential (25). A light-induced depolarization or a long train of action potentials may cause slow inactivation, thus effectively raising the firing threshold and halting firing, but without reversing the photomodification.

Modification of gating in unblocked channels also contributes to perturbations in firing behavior. The slowing of inactivation kinetics and the foot in the inactivation curve (Figure 2) both lead to a distortion in shape and considerable prolongation of action potential duration, up to hundreds of milliseconds in some cases. A very long action potential can itself serve as a prolonged stimulus to nearby axon segments that have received a lower light dose. In most experiments only a small segment is illuminated, thus permitting electrotonic interactions between the modified region and surrounding unmodified segments.

The finding that the closure of open channels is slowed, while the kinetics of conditioned inactivation remain normal, supports a previously stated contention (32) that normal inactivation may occur as two independent processes--one that occurs only following activation of channels, and one that is independent of activation.

Chemical mechanisms. Channel function is very easily perturbed by a variety of pharmacological agents that block and/or modify gating. The open pore region of a channel that discriminates between different ion species (the selectivity filter) is probably no more than a few Angstroms in diameter and it wouldn't take a gross structural abnormality in this part of a channel to block ion movement (25). The photochemical mechanisms underlying modification of axon function are not well understood, however. Voltage clamp experiments indicate that different sensitizers vary greatly in their potency, but bring about the same kinds of actions qualitatively, implying a common mode of action. (Lyudkovskaya described some sensitizer-specific actions in non-voltage clamped axons, but these have not been seen in voltage clamp experiments.) The major modification of sodium channels--block and perturbation of inactivation--are probably independent processes occurring in parallel. For a given degree of channel block, however, the perturbation of inactivation can vary somewhat depending on reaction conditions. For example, rose bengal, in contrast to other sensitizers, is so effective at sensitizing channel block that the relatively low light doses required to block 50% of the channels may not perturb inactivation measurably (28, 33). Thus sensitizer accessibility to a blocking site and an inactivation site may vary somewhat from sensitizer to sensitizer. An increase in leakage could result from modification of existing channels, creation of new pathways through other integral membrane proteins or by a perturbation in the lipid bilayer structure.

There are limited data pointing to singlet oxygen as an intermediate. On lobster axons deuterium oxide and azide were able to enhance and inhibit channel block by about 50% each, using rose bengal or eosin as sensitizers (34). On squid axons, with reagents perfused inside the cell, β-carotene effectively blocked sensitization by methylene blue but not rose bengal (28). Uncertainties in the distribution of reagents within the complex anatomy of a cellular system clouds the interpretation of these experiments.

Application to Light-Activated Pesticides

Since none of the investigation on photodynamic modification of excitable cells has been performed on insect species, any extrapolation to light-activated pesticides must of necessity be very speculative. However, if sensitizers in contact with insects can permeate to excitable membranes (i.e, they are not stopped by major diffusion barriers), it seems very likely that the kinds of modifications found in other life forms would also occur in insects.

Within a whole organism there are many photomodifiable sites. So long as the sensitizer is not photobleached then all sites will become modified at sufficiently high light doses. To be of phototoxic importance, however, some must exhibit phototoxic potential at relatively low light doses. Within a network of nerve cells an array of elementary processes can be perturbed. At a given light dose some will be far more modified than others because of different pre-illumination associations with sensitizer and different effective quantum yields. The consequence of a given

modification will also vary. (To use an analogy: A car can sustain
a crushed bumper better than a broken spark plug.) At some point
the accumulating modifications will become manifestly toxic and
survival of the organism threatened. Of the known photodynamic
perturbations in excitable cells, which ones have both a high
susceptibility to modification and a pivotal role in signaling? The
obvious choice, block of sodium channels, is probably not crucial
because of the large excess number of channels relative to the
minimum required to propagate action potentials. Only after many
other perturbations have occurred is it likely that enough sodium
channels would be blocked to halt propagation. Block of potassium
channels is also not likely to be crucial because of their
relatively low sensitivity to block. Interference with sodium
channel inactivation may be more important because the prolongation
of action poential duration that results from the interference
decreases the maximum frequency of firing during a burst of action
potentials. Even this, however, seems less important than light-
induced firing at neuromuscular junctions. In vertebrate species
each muscle cell is a "slave" to the motoneuron that innervates it
and light-induced firing translates directly into muscle
contraction. Extraneous muscle contraction interferes with
locomotion. In turn, the disturbed locomotion translates into
interference with feeding, escape from predators and reproduction.
Whether this occurs in insects remains unknown, however. While
neuromuscular transmission in insects has many mechanistic
similarities to that in vertebrates (35) the different transmitter
substance employed, method of transmitter removal, and the
difference in innervation pattern (18) makes this an open question.
         As noted in the introduction, one of the important elemental
processes occurring in all nervous systems is spontaneous generation
of excitation. The control of insect walking, in which muscles are
alternately stimulated and inhibited, is thought to originate in a
group of pacemaker cells that undergo rhythmic oscillations in
membrane potential (36). The frequency of oscillation is
continuously modulated by synaptic input. Such labile cells should
be easily perturbed by photodynamic means. While no photodynamic
studies have been carried out on insect pacemaker cells, it seems
very likely that these cells might be among the most susceptible to
the action of light-activated pesticides.

Literature Cited

1.   Callaham, M. F.; Lewis, L. A.; Holloman, M. E.; Broome, J. E.;
     Heitz, J. R. Comp. Biochem. Physiol. 1975, 51C, 123-128.
2.   Narahashi, T. Physiol. Rev. 1974, 54, 813-889.
3.   Chalazonitis, N. Thèse Sciences, Paris 1954, Ser. A, No. 2994,
     Order 3866, 1-116.
4.   Chalazonitis, N.; Chagneux, R. Bull. Inst. Oceanogr. (Monaco)
     1961, 58(1223), 1-20.
5.   Chalazonitis, N. Photochem. Photobiol. 1964, 3, 534-559.
6.   Lyudkovskaya, R. G. Biofizika 1961, 6, 300-306.
7.   Lyudkovskaya, R. G.; Kayushin, L. P. Biofizika 1959, 4, 404-
     412.

8. Lyudkovskaya, R. G.; Kayushin, L. P. Biofizika 1960, 5, 663-670.
9. Bostock, H. J. Physiol. 1982, 332, 57P-58P.
10. Kuffler, S. W.; Nicholls, J. G.; Martin, R. A. From Neuron to Brain, 2nd ed. Sinauer: Sunderland, MA, 1984.
11. Lippay, F. Pflug. Arch. 1929, 222, 616-639.
12. Lippay, F. Pflug. Arch. 1930, 224, 587-599.
13. Lippay, F.; Wechsler, L. Pflug. Arch. 1930, 224, 600-607.
14. Rosenblum, W. I. J. Cell. Comp. Physiol. 1960, 55, 73-79.
15. Burmistrov, Y. M.; Lyudkovskaya, R. G. Biofizika 1968, 13, 55-65.
16. Lyudkovskaya, R. G.; Pevzner, L. P. Biofizika 1964, 9, 580-588.
17. Sazonenko, M. K. Biofizika 1963, 8, 681-689.
18. Usherwood, P. N. R.; Cull-Candy, S. G. In Insect Muscle; Usherwood, P. N. R., Ed.; Academic: London, 1975; pp. 207-280.
19. Kohli, R. P.; Bryant, S. H. Experientia 1964, 20, 368-369.
20. Pooler, J. P. Biophys. J. 1968, 8, 1009-1026.
21. Pooler, J. P.; Oxford, G. S. J. Membr. Biol. 1973, 12, 339-348.
22. Adelman, W. J.; Adams, J. J. Gen. Physiol. 1959, 42, 655-664.
23. Hodgkin, A. L.; Huxley, A. F.; Katz, B. J. Physiol. 1952, 116, 424-448.
24. Hodgkin, A. L.; Huxley, A. F. J. Physiol. 1952, 117, 500-544.
25. Hille, B. Ionic Channels of Excitable Membranes. Sinauer: Sunderland, MA, 1984.
26. Pooler, J. P. J. Gen. Physiol. 1972, 60, 367-387.
27. Pooler, J. P.; Valenzeno, D. P. Photochem. Photobiol. 1979, 30, 491-498.
28. Oxford, G. S.; Pooler, J. P.; Narahashi, T. J. Membr. Biol. 1977, 36, 159-173.
29. Goldman, L. Q. Rev. Biophys. 1976, 9, 491-526.
30. Dubois, J. M. Prog. Biophys. Molec. Biol. 1983, 42, 1-20.
31. Pooler, J. P.; Valenzeno, D. P. Biophys. J. 1983, 44, 261-269.
32. Oxford, G. S.; Pooler, J. P. J. Gen. Physiol. 1975, 66, 765-779.
33. Pooler, J. P.; Valenzeno, D. P. Photochem. Photobiol. 1978, 28, 219-226.
34. Pooler, J. P.; Valenzeno, D. P. Photochem. Photobiol. 1979, 30, 581-584.
35. Pichon, Y. In Insect Neurochemistry and Neurophysiology; Borkovec, A. B.; Kelly, T. J., Eds.; Plenum: New York, 1984; pp. 23-50.
36. Shepherd, G. S. Neurobiology. Oxford: New York, 1983.

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:

Blum (<u>1</u>), Errera (<u>2</u>), Clare (<u>3</u>), Fowlks (<u>4</u>), Santamaria (<u>5-6</u>),
Santamaria and Pinto (<u>7</u>), Spikes and Ghiron (<u>8</u>), Simon (<u>9</u>), Spikes
and Straight (<u>10</u>), Bourdon and Schnurizer (<u>11</u>), Spikes (<u>12</u>),
Spikes and Livingston (<u>13</u>), Pooler and Valenzeno (<u>14</u>), and
Robinson (<u>15</u>).
     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 ... photosen-
sitized modification under the right conditions" (<u>14</u>). Sensitized
molecules are able to oxidize a wide range of cellular components
including intermediates, proteins, and membranes.
     Pooler and Valenzeno (<u>14</u>) cite several examples of photo-
chemical damage and modifications occurring to intracellular com-
ponents by photosensitizing agents. Once permeability of the cell
membrane has been altered, cellular function can be greatly
modified. Cande et al. (<u>16</u>) 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 (<u>17</u>) 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. (<u>18</u>) 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 susceptible to photosensitized
attack.
     Palm (<u>19</u>) 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 (<u>14</u>)) 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. (<u>20</u>) 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 (<u>Aedes</u> <u>triseriatus</u> (Say)).

Fondren and Heitz (<u>21</u>) observed LT_{50} and tissue levels of
dyes and suggested that the face fly (<u>Musca</u> <u>autumnalis</u> DeGeer) is
susceptable to toxic intracellular reactions of certain sensitized
xanthene dyes. Fondren et al. (<u>22</u>) conducted similar studies on
the house fly (<u>Musca</u> <u>domestica</u> 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. (<u>23</u>) reported that dye-sensitized photooxi-
dation of the acetylcholinesterase in whole-head homogenates of
the imported fire ant (<u>Solenopsis</u> <u>richteri</u> (Forel)) could be
induced in the presence of several xanthene dyes. Lactic dehydro-
genase and acetylcholinesterase of the boll weevil (<u>Anthonomus</u>
<u>grandis</u> <u>grandis</u> Boheman) were inactivated by dye-sensitized
photooxidation mediated by substituted xanthenes (<u>24</u>).

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 (<u>25</u>). Clark (<u>25</u>)
studied the mutagenic activity of several dyes in <u>Drosophila</u>
<u>melanogaster</u> 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 (<u>26</u>). Acriflavine and acridine orange were
reported to increase sex-linked and second chromosome recessive
lethal mutations in both males and females of <u>D</u>. <u>melanagaster</u>. In
the silkworm, <u>Bombyx</u> <u>mori</u> (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. (<u>27</u>) studied the effects of methylene blue on
fixed eukariotic chromosomes of the mosquito <u>Culiseta</u> <u>longiareo-</u>
<u>lata</u> 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 (<u>28</u>) 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 <u>Salmonella</u> and single-strand breaks in Chinese ham-
ster ovary cells (<u>29</u>). 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 photosensiti-
zers 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 photodyna-
mic 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 swel-
ling 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 diver-
sities 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 photodyna-
mic 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 noncircu-
lating 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 treat-
ment may have caused an increase in the number of adhering hemo-
cytes 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 obser-
vations 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 main-
tained 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 acetylcho-
linesterase 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 com-
pound 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 sclero-
tization 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% reduc-
tion 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% inhi-
bition 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 pho-
toactive 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 repro-
ductive 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.

Literature Cited

1. Blum, H. F. Photodynamic Action and Diseases Caused by
    Light; Reinhold Publ. Corp.: New York, 1941; 309 p.
    (Reprinted in 1964 with an updated appendix by Hafner Publ.,
    New York).
2. Errera, M. Progr. Biophys. Biophys. Chem. 1953, 3, 88-130.
3. Clare, N. t. In Radiation Biology; Holleander, A., Ed.;
    McGraw-Hill: New York, 1956; Vol. III, pp 693-723.
4. Fowlk, W. L. J. Invest. Dermatol. 1959, 32, 233.
5. Santamaria, L. In Recent Contributions to Cancer Research
    in Italy, Tumari Suppl.; Bucalossi, P.; Veroneri, U.,
    Eds.; Casa Editrice Ambrosiana: Milan, 1960; pp 167-287.
6. Santamaria, L. Bull. Sol. Chim. Belges. 1962, 71, 889.
7. Santamaria, L.; Pinto, G. In Research Progress in Organic,
    Biological and Medicinal Chemistry; Gallo, U; Santamaria, L.,
    Eds.; Soc. Editoriala Farmaceutica: Milan, 1964; Vol.
    3, pp 259-336.
8. Spikes, J. D.; Ghiron, C. A. In Physical Processes in
    Radiation Biology; Augenstein, L. G.; Mason, R; Rosenberg,
    B., Eds.; Academic Press: New York, 1964; pp 309-336.
9. Simon, M. I. In Comprehensive Biochemistry; Florkin, M.;
    Stotz, E. H., Eds.; Eleesevier: Amsterdam, 1967, Vol. 27, pp
    137-56.
10. Spikes, J. D.; Straight, R. Ann. Rev. Phys. Chem. 1967,
    18, 409-36.
11. Bourdon, J.; Schnurizer, B. In Physics and Chemistry of the
    Organic Solid State; Fax. D.; Labes, M. M.; Weissberger, A.,
    Eds.; Wiley (Interscience): New York, 1967, Vol. 3,
    pp 59-131.
12. Spikes, J. D. In Photophysiology III, Current Topics;
    Giese, A. C., Ed.; Academic Press: New York, 1968; pp 36-64.
13. Spikes, J. D.; Livingston, R. Adv. Rad. Biol. 1969, 3,
    29-121.
14. Pooler, J. P.; Valenzeno, D. P. Med. Phys. 1981, 8,
    614-28.
15. Robinson, J. R. Res. Rev. 1983, 88, 69-100.

16. Cande, W. Z.; McDonald, K.; Meeusen, R. L. J. Cell Biol.
    1981, 88, 618-29.
17. Haga, J. Y.; Spikes, J. D. Research Progress in Organic,
    Biological and Medicinal Chemistry; Galo, U; Santamaria, L.,
    Eds.; American Elsevier Publishing Co.: New York, 1972;
    Vol. 3, pp 464-79.
18. Hilf, R.; Smail, B. D.; Murant, S. R.; Leakey, B. P.;
    Gibson, L. S. Cancer Res. 1984, 44, 1483-88.
19. Palm, N. B. Ark. Zool. Stockholm, Series II 1952, 3,
    195-272.
20. Carpenter, T. L.; Mundie, T. G.; Ross, J. H.; Heitz, J. R.
    Environ. Entomol. 1981, 10, 953-55.
21. Fondren, J. E. Jr.; Heitz, J. R. Ibid. 1978, 7, 843-46.
22. Fondren, J. E. Jr.; Norment, B. R.; Heitz, J. R. Ibid.
    1978, 7, 205-8.
23. Callaham, M. F.; Lewis, L. A.; Holloman, M. E.; Broome,
    J. R.; Heitz, J. R. Comp. Biochem. Physio. 1975, 51C,
    123-28.
24. Callaham, M. F.; Palmertree, C. O.; Broome, J. R.; Heitz,
    J. R. Pestic. Biochem. Physiol. 1977, 7, 21-7.
25. Clark, A. M. Am. Nat. 1953, 87, 295-305.
26. Nasim, A.; Brychcy, T. Mutation Res. 1979, 65, 261-88.
27. Bianchi, U.; Mezzanotte, R.; Ferrucci, L.; Marshi, A. Cell
    Differentiation. 1980, 9, 323-28.
28. Gruener, N.; Lockwood, M. P. Biochem. Biophys. Res. Commun.
    1979, 90, 460-65.
29. Nestmann, E. R.; Douglas, G. R.; Matula, T. I.; Grant, C. E.;
    Kowbel, D. J. Cancer Res. 1979, 39, 4412-17.
30. Ben-Hur, E.; Prager, A. Green, M. Rosenthal, I. Chem.-Biol.
    Interact. 1980, 29, 223-33.
31. Towers, G. H.; Abramowski, Z. J. Nat. Prod. 1983, 46,
    572-77.
32. Weaver, J. E.; Butler, L; Amrine, J. W. Jr. Environ.
    Entomol. 1982, 11, 463-66.
33. Yoho, T. P. Ph.D. Thesis, West Virginia University, West
    Virginia, 1972.
34. Schildmacher, H. Biol. Zentr. 1950, 69, 468.
35. Respicio, N. C.; Heitz, J. R. Bull. Environ. Contam. Toxicol.
    1981, 27, 274-81.
36. Carpenter, T. L.; Heitz, J. R. Environ. Entomol. 1980, 9,
    533-37.
37. Broome, J. R.; Callaham, M. F.; Poe, W. E.; Heitz, J. R.
    Chem.-Biol. Interactions 1976, 14, 203-6.
38. Callaham, M. F.; Broome, J. R.; Poe, W. E.; Heitz, J. R.
    Environ. Entomol. 1977, 6, 669-73.
39. Weaver, J. E.; Butler, L.; Yoho, T. P. Ibid. 1976, 5,
    840-44.
40. Kondo, M.; Kasai, M. Photochem. Photobiol. 1974, 19, 35-41.
41. Levitan, H. Proc. Natl. Acad. Sci. 1977, 74, 2914-18.
42. Augustine, J. G.; Levitan, H. J. Physiol. 1983, 334, 65-77.
43. Selverston, A. I.; Kleindienst, H. U.; Huber, F. J. Neurosci.
    1985, 5, 1283-92.
44. Yoho, T. P.; Weaver, J. E.; Butler, L. Environ. Entomol.
    1973, 2, 1092-96.

45.   Broome, J. R.; Callaham, M. F.; Lewis, L. A.; Ladner, C. M.;
      Heitz, J. R.   Comp. Biochem. Physiol.   1975, 51C, 117,21.
46.   Barbosa, P.; Peters, T. M.   Histochemical J.   1971, 3, 71-93.
47.   Callaham, M. F.; Broome, J. R.; Lindig, O. H.; Heitz, J. R.
      Environ. Entomol.   1975, 4,   837-41.
48.   Sakurai, H.; Heitz, J. R.   Ibid.   1982, 11, 467-70.
49.   Clement, S. L.; Schmidt, R. S.; Szatmari-Doogman, F.;
      Levine, E.   J. Econ. Entomol.   1980, 73, 390-92.
50.   Zacharuk, R. Y.   Can. J. Zool.   1963, 41, 991-96.
51.   Bridges, A. C.; Cocke, J.; Olson, J. K.; Mayer, R. J.
      Mosq. News.   1977, 37, 227-31.
52.   Pimprikar, G. D.; Norment, B. R.; Heitz, J. R.   Environ.
      Entomol.   1979, 8, 856-59.
53.   Fairbrother, T. E.; Essig, H. W.; Combs, R. L.; Heitz, J. R.
      Ibid.   1981, 10, 506-10.
54.   Downum, K. R., Rosenthal, G. A., Towers, G. H. N.   Pestic.
      Biochem. Physiol.   1984, 22, 104-9.
55.   David, J.   C.r. Acad. Sci., Paris.   1955, 241, 116-18.
56.   David, J.   Bull. Biol. France Belgique.   1963, 97, 515-30.
57.   Pimprikar, G. D.; Noe, B. L.; Norment, B. R.; Heitz, J. R.
      Environ. Entomol.   1980, 9, 785-88.
58.   Kagan, J.; Chan, G.   Experientia.   1983, 39, 402-3.

Chapter 9

Multiple Mechanisms of Dye-Induced Toxicity in Insects

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Several xanthene dyes have been proven to be toxic
to various species of insects. Three types of toxic
mechanism have been observed in insects namely: a
light dependent mechanism, a light independent mecha-
nism and a developmental toxicity mechanism. The
light dependent mechanism is quite fast and involves
production of singlet oxygen. The dark reaction
is comparatively slow. Both reactions cause
histological, behavioral, physiological, and biochem-
ical changes in insects. Several morphological
abnormalities are caused by the dye treatment in
various insect species. Xanthene dye treatment also
affects growth and development in insects. Attempts
have been made to review the biochemical, physio-
logical, and developmental aspects of these
multiple toxicity mechanisms.

Several synthetic dyes and natural products are known to be
toxic to various agricultural and public health insect pests.
After extensive field testing, one of the synthetic dyes, erythro-
sin B, has been registered by the Hilton-Davis Chemical Company for
house fly control in caged layer chicken houses under the name
Intercept or Synerid.

In earlier days, the toxicity was thought to be due to the
production of singlet oxygen and that light was an essential ele-
ment for the toxicity. However, work done by various researchers
over the last decade has shown that there are three types of toxi-
city mechanisms associated with these compounds:

1. Light dependent toxicity mechanism
2. Light independent toxicity mechanism
3. Developmental toxicity mechanism

The light dependent toxicity mechanism is quite fast and needs comparatively lower concentrations of the photosensitizer and a source of light. The light independent or dark mechanism is slow, needs a higher concentration of sensitizer and operates in the absence of light. In the developmental toxicity mechanism, the insect is exposed to a sublethal dose of the compound in the earlier stages of development. This results in mortality or some adverse morphological abnormalities during development, such as delayed development, growth retardation, and fecundity and fertility changes.

Light Dependent Toxicity Mechanisms

The light dependent toxicity mechanism (or photodynamic action) in insects involves the ingestion of the photosensitizer by the insect, followed by exposure to a visible light source which results in the death of an insect. Several synthetic and natural compounds have been reported to act as effective photosensitizers in biological systems including xanthenes, acridines, phenothiazines, psoralens, flavins, porphrins, quinones, polyines and thiophenes.

Photodynamic action involves photooxidation of various substrates which results in inactivation of biological systems, distortion of membranes, inactivation of enzymes, cell death and other special function losses (1-4). Photodynamic action occurs via either a "Type I" mechanism which involves electron transfer reactions or a "Type II" mechanism which involves singlet oxygen (5). In heterogenous biological systems the photodynamic reaction may not be strictly Type I or Type II mechanism but it could involve both mechanisms. Foote has reviewed the Type I and Type II mechanisms, the factors determining the efficiency, and the relative participation of these mechanisms in an earlier chapter of this book.

The photodynamic damage in vivo may occur wherever an efficient photosensitizer can be intimately deposited in an actively respiring medium and can receive adequate illumination (6). In insects, photodynamic damage most probably occurs in the membranes of the gut wall followed rapidly by implication of other lipoidal membranes as the highly lipid-soluble photosensitizer diffuses throughout the organism. The permeability of the photosensitizer into the cell, distribution of the photosensitizer among various cell components, and binding of the photosensitizer to the substrate determines the nature and extent of the photodamage (7).

Actual membrane penetration by the photosensitizer itself may not be required to produce high lethality when the contact is sufficiently intimate and involves a large specific surface (8). The singlet oxygen produced in photodynamic action can freely diffuse miceller as well as aquous phases and can react

with organic substrates at different sites (9-10). Considering the reactivity of the singlet oxygen with unsaturated lipids, it indicates that transmembrane diffusion could scarcely take place in the absence of some undefined transport mechanism without pertubation of membrane transport (8). Nieumint et al (11) recently presented evidence that the interaction of photosensitizer and substrate does influence actual product formation. Direct interaction could take place between the sensitizer and adjacent residues while more distant domains could be oxidized by diffusable intermediates such as singlet oxygen (12).

Photodynamic action is known to cause nuclear, ribosomal, cytoplasmic, and cell membrane damaging reactions leading ultimately to cell death. The three primary target sites of the photodynamic action are:

1. Biochemical components
2. Biological membranes
3. Vital enzyme systems

1. Biochemical Components

The effect of photodynamic action on various biochemical components has been reviewed by Spikes in an earlier chapter of this book. In order to avoid duplication, this area is very briefly summarized here. The biochemical functional groups which are attacked by photodynamic action include proteins, carbohydrates, steroids, amino acids (cysteine, tryptophan, histidine, tyrosine, and methionine), fatty acids, nucleic acids, thiols, sulfides, and disulfides (13).

Binding of the dye to biological macromolecules is crucial and may affect the relative efficiency of Type I and Type II pathways for photooxidation available to the sensitizer (14). Secondly, photodynamic effects in vivo are largely dependent on the site to which the photosensitizer binds. Rose bengal binds at hydrophobic sites and lyses membranes while acridine orange penetrates to the nucleus and causes damage to DNA (7,15-16).The furanocoumarins also bind and create photochemical damage at the level of DNA (17).

The photosensitized oxidation of proteins, as well as other biochemical components, alters or destroys normal biological functions. In the case of proteins, photoalteration is due to the degradation of the side chains of five amino acids. Inactivation results from the destruction of essential amino acid residues at or near the active site or binding site of the enzyme and/or by the degradation of residues elsewhere that are required for the

maintainance of the appropriate catalytic conformation of the
molecule (13). The illumination of photosensitizer-protein mixtures
can result in the formation of covalent sensitizer-protein photoad-
ducts which can alter the properties of proteins.

Photodynamic action on nucleic acids results in selective
destruction of guanine residues (18) and alteration of the physical
properties of DNA. Photodynamic action sensitized by rose bengal
can cause strand breaks in DNA. The sensitizer and oxygen molecules
interact with the DNA so as to effect site-specific singlet oxygen
generation which causes photodynamic lesions (11). The photosen-
sitized oxidation of residues in template DNA and RNA decreases the
efficiency of transcription and translation respectively (4).
Naturally occuring photosensitizers, such as Khellin and the furano-
coumarins, form monofunctional adducts resulting in interstrand
cross-linkage of DNA in various developmental stages of insects
(17,19-21). These photodynamic damages have serious consequences
for DNA transcriptions and can lead to cell death or mutagenesis.

Photodynamic action affects biologically important lipids in
the form of unsaturated lipids, such as fatty acids, triglycerides
and phospholipids; and unsaturated lipid-soluble biomolecules, such
as cholesterol, vitamin D, sterols, steroids, and prostaglandins
(13). Lipid peroxidation is quite destructive to biological membra-
nes. This topic is discussed in detail in the following section on
the effect of photodynamic action on biological membranes.

Recent studies showed a depletion of glutathion levels due to
the photodynamic action in insects (22-23). Wages (22) also
recorded a depletion in NADPH levels accompanied by a moderate
increase in NADP levels in the photodynamically treated house flies.
The author suggested that, assuming some relation between the deple-
tion of NADPH and glutathione, at least some of the glutathione is
being oxidized to glutathione disulfide, since the major enzyme
involved in maintaining the equilibrium between glutathione and glu-
tathione disulfide, glutathione reductase, utilizes NADPH as a donor
of electrons for the reduction of glutathione. Photodynamic action
may result in depletion of important biochemical groups which are
indispensable to the insect from the viewpoint of energy metabolism
or detoxification mechanisms.

2. Biological Membranes

Valenzeno and Pooler have reviewed the effects of photodynamic
action on biological membranes in earlier chapters of this book.
Various histological abberations due to photodynamic action have
been reported in the literature, both with synthetic and naturally

occuring photosensitizers, which result in physiological changes in
insect systems. The midgut wall and the crop of the dye-fed, light
exposed house fly and mosquito have been observed to suffer cellu-
lar damage. The gut was reported to be distended with numerious
air bubbles (24-25) suggesting an alteration in the membrane struc-
ture which probably causes changes in membrane permeability and
lysis of cellular organelles in the midgut epithelium.

    Volumetric changes in the haemolymph and crop contents of
cockroaches were observed due to photodynamic action (26). These
changes appear to reflect a transfer of haemocoel fluids into the
alimentary canal and perhaps into tissue. The dyes may affect the
permeability of cell membranes thereby creating a differential in
osmotic pressure which allows hemocoel fluids to pass into the ali-
mentary canal. A substantial decrease in haemolymph volume over a
relative short period of time may contribute to the death of the
insects.

    Histological and physiological damage is probably due to photo-
dynamic action on biological membranes and the singlet oxygen
mechanism is suspected in many cases. The physiological and histo-
logical effects of photodynamic action have been reviewed by Weaver
in the previous chapter of this book. It seems that photodynamic
action alters the membrane protein as well as lipid components of
biomembranes (lipid bilayer). Sodium channels are blocked and the
permeability to potassium ions is affected (27-31). The altered
membrane structure and changes in the membrane permeability may
lead to cell death.

    Freeman and Giese (32) reported that rose bengal initially
forms a complex at the cell membrane in yeast cells. Illumination
leads to binding and photooxidation, first at the surface and then
in the cytoplasm, as the dye diffuses inwards. Singlet oxygen
passes through the cell membrane and diffuses into the cytoplasm
producing damage along its path to the membrane leading to photo-
haemolysis of the cells. Pooler and Valenzeno (33) studied
photochemical damage occuring to intracellular components by photo-
sensitizing agents. The rose bengal binds on the outer membrane
surface with its two negative charges exposed to the aqueous medium
and the hydrophobic portion of the molecule inserted in the lipid
bilayer. Photodynamic lesions are created when membranebound dye
molecules generate active oxygen. Photoxidative damage to cell
membranes leads to leaching of potassium out of cells and then to
cytoplasmic extrusion (34). The permeability of the cell membrane
is altered which results in modification of cellular function.

    The lysed cells seem to be permeable to erythrosin B (35).
Several workers observed holes in the plasma membrane and
mitochondria appear to be swollen and distorted (35-36).

The swelling is probably due to the inhibition of enzymatic activities in the elecron transport system and by uncoupling of phosphorylation from respiration (36).

The photodynamic effects of cercosporin showed the changes associated with lipid peroxidation (37). There was an increase in the ratio of saturated to unsaturated fatty acids, and a decrease in the fluidity of the membrane resulting in changes in membrane permeability. Electrolyte leakage and cell death may be accounted for these perturbations of membrane composition and structure. Photodynamic damage is dependent on the fatty acid composition of the membranes and the osmolarity of the medium (38). Ito (39) proposed two modes of cell-dye interaction; i.e., membrane attack by extracellularly generated singlet oxygen and attack by the dye localized in the hydrophobic region of the membrane.

3. Vital Enzyme Systems

Photodynamic action has been observed to cause inactivation in several groups of enzymes including the enzymes crucial to metabolic pathways such as glycolysis, the Krebs cycle, amino acid metabolism, pentose phosphate pathway, fatty acid metabolism and oxidative phosporylation (4,40-42). The vital enzyme systems affected by photodynamic action include mixed function oxidases (43); cytochrome P-450 (44); alcohol dehydrogenases and lipoamide dehydrogenase (45-46); glucose-6-phosphate dehydrogenase (47); citrate synthetase (48); ATPase and adenyl kinase (49); acetylcholinesterase (50-53); and lactic dehydrogenase (54).

The most extensively studied insect enzyme system with photodynamic action is the acetylcholinesterase system which is vital for neurotransmission. Initial observation in dye-fed, light-exposed boll weevils and house flies showed hyperexcitation an increased activity (24). An attempt has been made to quantitate the locomotary activity of dye-treated and control house flies using a vibration sensitive actograph system (Table I).

Table I. Effect of Rose Bengal Treatment on the Locomotary Activity of House Fly, M. domestica

| Conditions | Locomotary Activity[a] [b] | | Percent Difference in Activity |
	Control	Treated	
Room light	404.6>25.3	587.6>35.7	45.25[c]
Dark	206.4>22.8	217.9>25.6	5.55
Night	13.4> 1.4	13.9> 1.9	3.52

[a]Activity in units per hour for 25 females
[b]Mean of 51 replicates > SE
[c]Statistically significant at 0.05% level

The rose bengal-treated, light-exposed insects showed about 45 percent increased locomotary activity relative to control flies. The symptoms of photodynamic toxicity, such as increased irritability, increased antennal grooming, and increased locomotary coordination followed by paralysis and death (24) clearly indicate the involvement of the nervous system. Several researchers observed the inactivation of acetylcholinesterase due to photodynamic action (24,52).

In summary, the singlet oxygen generated in photodynamic action is an indiscriminate oxidizing agent such that there may not be one single critical target site affected at one time. Death may occur in insects as a cumulative effect of the oxidation of many discrete targets.

II. Light Independent Toxicity Mechanism

The light independent toxicity mechanism (or the dark reaction) in insects operates in the absence of light. The concentration of toxic compound needed for the dark reaction is comparatively high and the time required for the lethal action is comparatively longer relative to the light dependent mechanism. This mechanism has been observed with several insect species both with synthetic dyes as well as with natural products.

The light independent toxicity of the xanthene dyes was first investigated by Blum (55). More recently it has been reported with the xanthene dyes in fire ants (56), boll weevils (53-54), face flies (57), house flies (58), corn ear worms (59), and mosquitoes (60). In the beginning it was thought that the light independent toxicity in insects is due to an organochlorine type of toxicity (57) which results in symptoms of energy stress. But the high levels of dark toxicity reported in the house flies with the non-halogenated dyes such as rhodamine B and rhodamine 6G (61) cast doubt on this hypothesis.

The light independent toxicity with natural products like alpha terthienyl, phenyl heptatriene, and xanthotoxin was reported in mosquito, black fly, Manduca, and Spodoptera larvae (62-65). The target in the dark reaction with the natural products appears to involve membranes (31,66) The Manduca larvae fed with the alpha terthienyl frequently produced liquid frass which indicates that the hind gut is failing to reabsorb water (65) and this may be due to the disruption of the epithelial membrane of the midgut and by interference with the function of the rectal glands (67).

The biochemical changes associated with the light independent
toxicity with the rose bengal were studied in the boll weevil
by Broome et al (53). Boll weevils fed for 4 days with rose bengal
were 18 percent lighter by wet weight and 41 percent lighter by dry
weight than control weevils. They contained 90 percent less lipid
and 41 percent less protein. Amino acid pools fluctuated drasti-
cally. In related studies, Callaham et al (54) observed that rose
bengal fed boll weevils did not lose weight or decrease protein
levels; rather, they remained constant, whereas the control insects
increased. Studies by Waldbauer (68) also indicated that in
treated insects, nutrients are diverted to repair damage and do not
contribute to growth.

Champaigne et al (69) reported that alpha terthienyl reduces
the gross efficiency with which the diet is converted to insect
biomass. Jorden and Smith (70) suggested that xanthene dyes inhi-
bit several well known detoxification systems and this may play an
important role in the light independent toxicity.

In summary, death by the light independent toxicity is probably
due to interference with the growth and survivorship of an insect
by disrupting the metabolic process, disrupting the epithelial
membranes of the gut, by interfering with nutrient assimilation or
by deterring feeding (69) which results in a lethal energy stress.

III. Developmental Toxicity

During the last decade, researchers from several laboratories
have observed and emphasized the adverse effects of photoactive
compounds on the development of insects. In the developmental
toxicity, earlier stages of the insects are exposed to sublethal
doses of the photoactive compounds and this results in either mor-
tality or some adverse effect in a later stage of development.
These adverse effects include formation of morphological abnor-
malities, growth retardation, prolonged developmental periods,
undersized individuals, and effects on fecundity, fertility, and
the sex ratio in insects. These developmental effects have been
observed both with synthetic dyes and natural products. These
effects are seen in either the presence or absence of light. The
concentration of the photosensitizer needed for developmental toxi-
city is comparatively low.

A. Morphological Abnormalities

Several morphological and physiological abnormalities in
response to treatment by photosensitizer during the development of
insects have been observed. The species of insect showing these
morphological abnormalities include Drosophila (71), alfalfa but-
terfly, Colias eurytheme (72), mosquito (73-75), face fly (76);
house fly (Pimprikar, unpublished); Papilio butterfly (77),

tobacco horn worm, Manduca sexta (69,78), and fire ant (Pimprikar, unpublished).

The level of morphological abnormalities induced is dependent on various factors such as concentration of the sensitizer length of exposure, presence of light, stage of insect, mode of application of the sensitizer, and the species of insect used in the experiment. Many of the morphological abnormalities resemble the effects induced by the juvenile hormone analogs or the chitin synthesis inhibitor such as Dimilin.

During the larval period, many of the insects survive the dye treatment at lower concentrations and remain outwardly unaffected until molting begins. Various morphological abnormalities observed in house flies, mosquitoes, face flies and fire ants are shown in Figure 1.

In the case of house fly and face fly larvae, the anterior and posterior regions exhibited pupation but the central region remained as larvae (Fig. 1A). These individuals could not survive beyond pupation and died in that stage.

In the case of mosquitoes, the treated larvae were unable to shed the old cuticle from the abdomen and head region. The partially shed exuvium remained attached to the larvae. Some larvae struggled laboriously to shed the exuvie but failed and eventually died in the process (Fig. 1B). There were several morphological intermediates observed with pupal head capsule and larval abdominal segments. Some pupae retained the 4th instar cuticle but those that pupated successfully often died later.

Failure of proper adult eclosion is the most prevelent of all the effects noted. The failure of adults to emerge completely from the puparium varied from complete lack of eclosion to only slight attachment of the wing or leg to the puparium (Fig. 1C). In the majority of cases, only the head emerged from the puparium. In other cases, the emerging adult was successful in separating body parts up to the thorax or even the legs and half of the abdomen from the pupal exuvium. Sometimes, the adult essentially comes out of the puparium but is still attached by various appendages and cannot free itself completely.

In many instances successfully emerged adults are not as healthy or active. Many of them appear to be small in size (79). The wings of successfully emerged adults may be curled, short, and non-functional (Fig. 1D, Fig. 1E) as seen in the mosquito (75), face fly (76), fire ant and house fly (Pimprikar, unpublished).

Morphologically normal face flies which emerged from erythrosin B-treated manure were shown to have a shorter life span than those emerged from control manure (79). This toxicity is
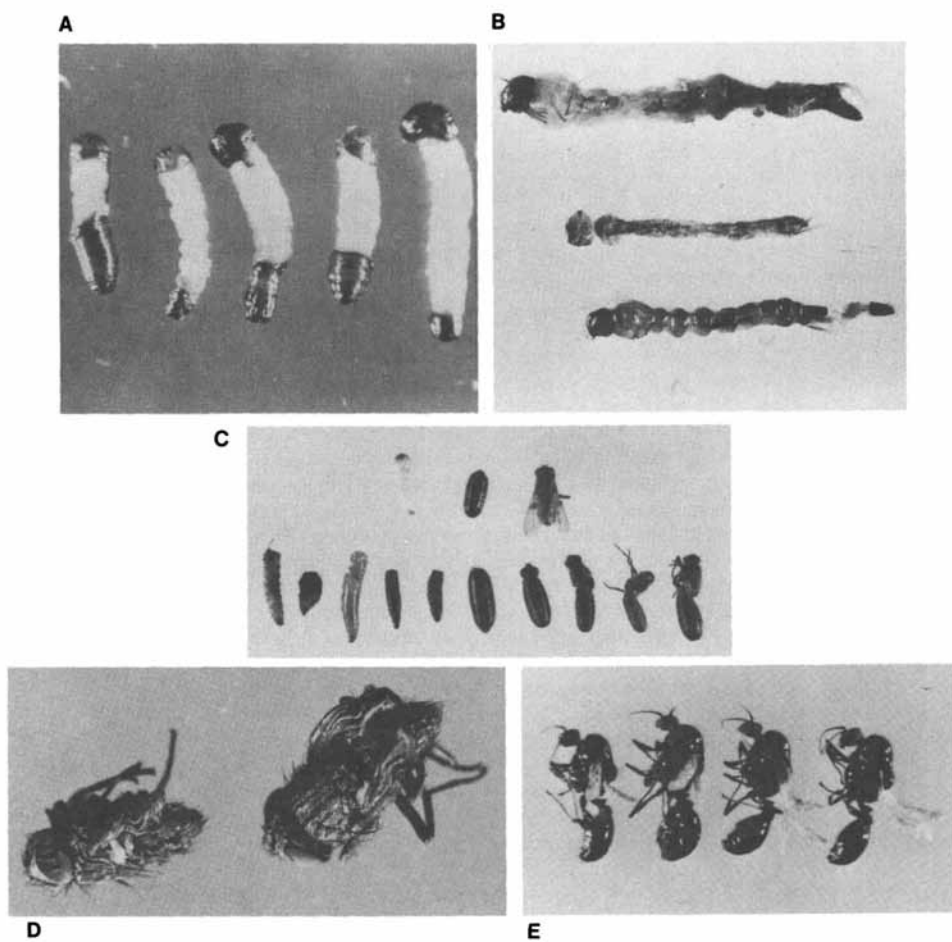
Figure 1.   Various morphological abnormalities observed due to the dye treatment in (A) house fly, (B) mosquito, (C) house fly, (D) deformed wings in face fly, and (E) deformities in wing in fire ants.

probably due to the effects of the residual dye levels consumed in
the larval stage and maintained in the tissue through the pupal
stage into the adult stage.

Quantitative studies on the effect of sensitizers on adult
emergence and also on morphological abnormalities were carried out
in mosquitoes (73), face flies (79), Pimprikar (unpublished). The
data in Table II indicates that the abnormalities as well as adult
emergence is dependent on the concentration of the photosensitizer.

Table II.   Effect of Rose Bengal and Erythrosin B on House
Fly Development[a]

Treatment	Percent Reduction in Adult Emergence[b]		Percent Abnormal Pupae[c]	
	RB	EB	RB	EB
Control	-	-	-	-
22 ppm	26.4	22.6	3.6	3.8
44 ppm	40.3	39.5	14.7	23.7
110 ppm	64.0	54.2	21.6	21.6

[a]Average of three replicates
[b]Percent reduction compared to control
[c]Corrected for control by Abbott's formula

Various researchers attempted to explain these morphological
abnormalities. Many of these problems seem to be associated with
normal muscle attachment. It seems that the enhanced mortality, as
well as abortive molting, may be due to the effects of the exertion
required at the emergence on a weakened insect. The treatment of
the photosensitizers results in a decrease in the weight of the
insect, reduction in total lipid and protein contents (53-54).
The photosensitizers are also capable of causing several biochemi-
cal changes in the insect system which could lead to stressful
development of an individual. These weakened insects probably can-
not resist muscular tension and increased turgor pressure during
the process of molting which may result in abortive molting.

Champaigne et al (69) reported that the partially molted
cuticle constricts the larvae of M. sexta when fed with the photo-
sensitizer. This prevents the passage of the gut contents and
restricts the circulation of the haemolymph. Eventually, the
anterior part of the larvae becomes turgid and the larvae stops
feeding and finally dies.

Downum et al (78) observed that the abnormalities in M. sexta
larvae caused by ingestion of alpha terthienyl are similar to the
abnormalities caused by the action of L-dopa in the southern army
worm as reported by Rehr et al (80). According to Rehr, the
deformed pupation might be due to the interference of tyrosinase,

which is an essential enzyme for hardening and darkening of the cuticle, by the non-protein amino acids. Downum et al (31) recorded in E. coli that the singlet oxygen, produced by UV-A activated alpha terthienyl, cross-links the membrane proteins. They speculate that similar effects caused by singlet oxygen in the integument of M. sexta might be responsible for the deformities in sclerotization.

Most abnormalities appear to be associated with problems in molting which lead to the hypothesis that these photosensitizers may have an effect on the molting hormones. The two most prominant molting hormones in insects are alpha-ecdysone (ecdysterone) and beta-ecdysone (20-hydroxyecdysterone). These hormones are steroidal in nature and the titers of these hormones control the sequence of developmental events such as molting, pupation, adult development and oogenisis (81).

An HPLC procedure for the quantitative determination of these two steroid hormones was reported by Pimprikar et al (82). The titers of ecdysterone and 20-hydroxyecdysterone during the development of the control and erythrosin B-treated house flies are shown in Figure 2A and Figure 2B. The titers of the hormones as well as the ratio of alpha- and beta-ecdysones are distinctly different in the erythrosin B-treated insects as compared to the control insects. It is thought that the imbalance of the molting hormone titers during the critical stages of development may contribute to the abortive molting or to the development of morphologically abnormal individuals.

An important factor which needs further consideration is the observation that some larvae successfully pupated and of these some successfully emerged as abnormal or normal adults. This might be due to an inability to select larvae for treatment with the photosensitizer which were in completely synchronous development. It also suggests that there are specific "developmental time windows" only through which the photosensitizer can be effectively introduced to cause morphogenetic effects.

B. Delayed Developmental Periods

Other developmental toxicity effects of the photosensitizers are reflected by the significant delays in developmental periods in insects. Two interrelated areas of interest with the delayed developmental period include the antifeedant activity of the photosensitizers and the development of smaller sized individuals.

Early research by Edwards (83) reported the retardation of growth in silkworm larvae fed on leaves sprinkled with methylene blue. In this instance, the author suggested that the low palatability of the dyed leaves may have caused the retardation of larval growth. Koyler (72) reported that the growth of the alfalfa caterpillar, Colias philodice and C. eurytheme, was prolonged when
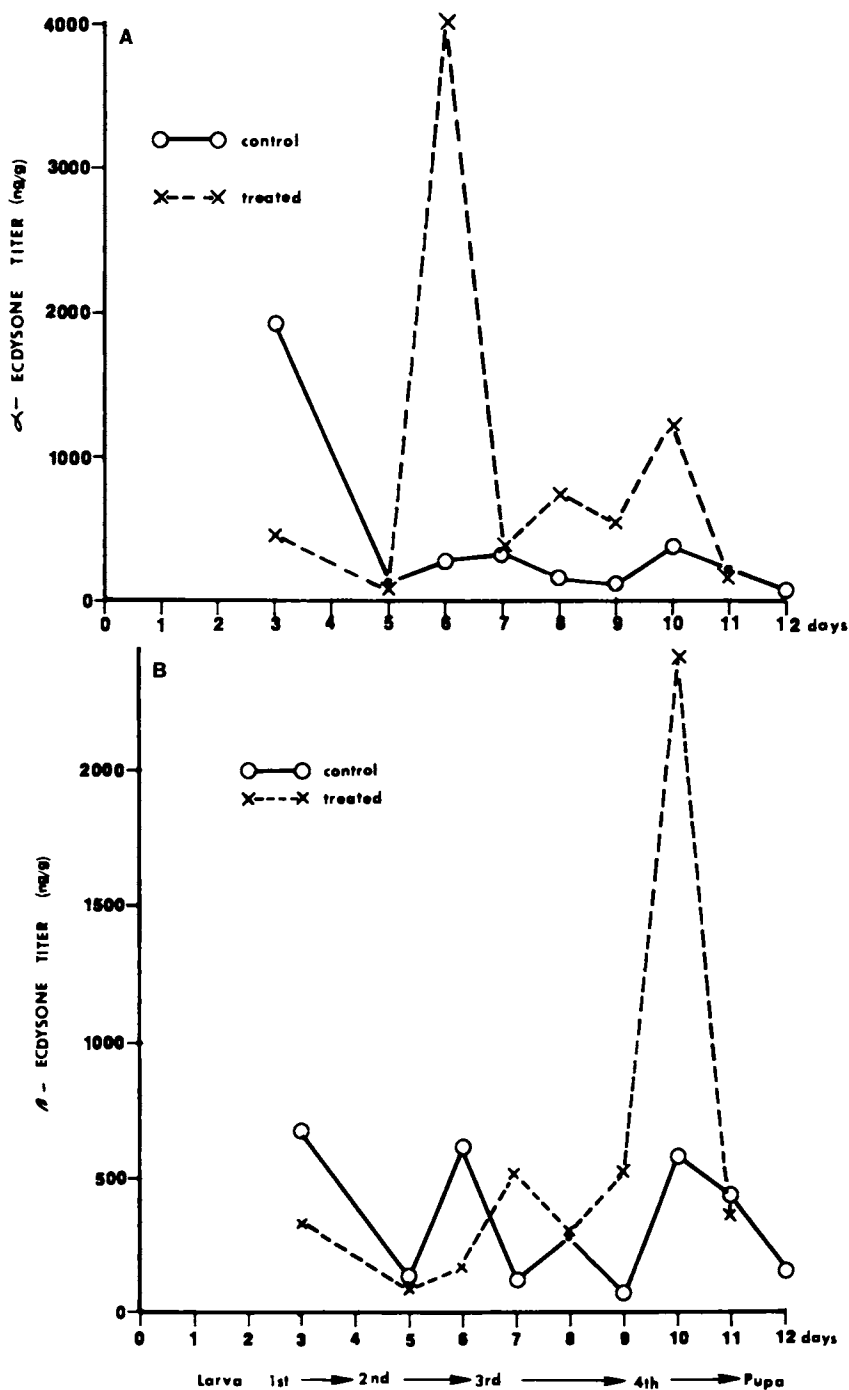
Figure 2.   Titers of (A) alpha-ecdysone and (B) beta-ecdysone during the development of house fly.

exposed to neutral red. David (71) observed that methylene blue can retard the growth of Drosophila larvae and that the retardation ranged from 17 to 400 hours with increasing concentration of dye.

Barbosa and Peter (84) demonstrated in a series of experiments, retardation of growth in the larvae of the mosquito, Aedes aegypti exposed to methylene blue and neutral red. The number of hours required for 50 percent or more larvae to pupate increases dramatically. The retardation in some cases was approximately 10 times that of control. In all the experiments attempting to illustrate the retardation of growth, they used the following three criteria:

1. Delay in onset of pupation
2. Length of larval period
3. Relative rates of pupation

The delay in the period of development was concentration dependent. The effect of exposure seemed to be less severe on the later instars. The authors concluded that the length and the stage of exposure may have a key role in the effects of dyes. They also conducted experiments to determine if there were any differences in the amount of food (yeast suspension) that the mosquito larvae would ingest when placed in various concentrations of dye. The main reason for this experiment was the possibility that retardation of growth might have been caused simply by lack of feeding due to unpalatable food. There was no significant difference in average larval weights indicating that the retardation of growth was not caused by rejection of dyed food under the conditions of the experiment.

Clement et al (85) also observed the retarded larval growth in the black cut worm. However, there was a remarkable decrease in the number of fecal pellets in the dye-treated larvae which indicated that the larvae consumed relatively smaller amounts of dye-treated food. Quantitave studies on the delayed developmental periods in the house fly due to erythrosin B and rose bengal treatment were conducted in our laboratory. The data in the Table III indicates that the larval and pupal periods were prolonged which were ultimately reflected in a corresponding delay in adult house fly emergence. There was a delay of about 3 to 4 days in the adult emergence in house flies reared on the dye-treated medium and the developmental delay was dependent on the concentration of the dye (Table III).

Table III.   Delayed Adult Emergence Due to Erythrosin B
Treatment in House Fly

Emergence on	Cumulative percent Adult Emergence[a]		
Day	Control	22PPM	110PPM
1	33.5	7.1	0.7
2	75.2	37.4	20.6
3	95.3	87.9	52.9
4	97.7	94.2	89.4
5	99.9	99.9	99.9

[a]Average of three replicates

The delayed developmental effects of the photosensitizers have
been studied recently by various researchers. Berenbaum and Feeny
(77) studied the toxicity and developmental effects of the linear
and angular furanocoumarins on the Papilio butterflies. The larvae
fed on leaves containing angelicin grew more slowly and weighed
less at pupation. The authors correlated the reduced pupal weights
with the reduced adult body size and concluded that the dele-
terious effect is due to ingestion of angelicin and not due to
reduced consumption. Downum et al (78) administered alpha
terthienyl to the tobacco horn worm, through an artificial diet and
observed a delay in pupation of the larvae. Similarly Kagan et al
(86) also reported prolonged larval periods in the mosquito due to
alpha terthienyl treatment.

Alpha terthienyl and phenyl heptatriyne are known to be potent
feeding inhibitors in several insect species like the European corn
borer, the cut worm, the tobacco budworm, and the colorado potato
beetle (69,87-88). The larvae of M. sexta consumed little diet and
produced few fecal pellets and it was suggested that starvation
contributed to mortality (69). Their studies also demonstrated
that photodynamic plant products can lenghten larval development
time, reduce growth, decrease the efficiency of conversion of
ingested food, and the efficiency of conversion of digested food.
Antifeedent activity experiments clearly indicated that alpha
terthienyl reduces feeding activity.

The net effect of the antifeedent activity of photosensitizers
probably results in the development of smaller sized individuals as
demonstrated by David (71) in Drosophila, Koyler (72) in the
alfalfa caterpillar and by Berenbaum et al (77) in Papilio
butterflies.

Barbosa and Peters (84) observed that female pupal weights in
A. aegypti decreased significantly due to the treatment of photo-
sensitizer. However, male pupal weights were not affected. They
proved experimentally that this was not caused by rejection of dye-
impregnated food. Sakurai and Heitz (89) reported decreased pupal
weights in the rose bengal- and erythrosin B-treated house flies.

The larvae of the cut worm, Euxoa messoria showed significantly depressed growth due to alpha terthienyl feeding. The larval and pupal weights were decreased by about 30 percent (69).

The delayed developmental periods and the antifeedent properties of the photosensitizers with the resulting effects on retardation of growth have profound implications in the practical application of the photosensitizers in integrated pest control programs in the following ways:

1. The number of generations per season could be reduced due to the prolonged growth periods.

2. Since the larval and pupal periods take longer for development, it gives additional time for parasites, predators, and natural enemies for effective control in the field (especially the harmless pupal stages).

3. The growth retarded individuals are likely to experience a substantial reduction in fitness compared to the normal insects.

According to Lewontin (90), even small changes in the development time can have great effects on reproductive potential. There is a need for further research on the mechanisms by which the retardation occurs so that it can be more precisely exploited for insect control.

C. Biotic, Ovicidal, and Other Effects

During the last decade, studies have indicated that naturally occuring and synthetic photosensitizers are both capable of causing deleterious biotic effects in insects. This includes effects on fecundity and fertility.

Fecundity represents the number of eggs laid by the female over her entire lifetime and fertility represents the viability of the laid eggs by the females. David (71,91) for the first time observed that fecundity in Drosophila was markedly lower due to the methylene blue treatment. Pimprikar et al (92) demonstrated the effect of rose bengal on fecundity and fertility in the house fly. Fecundity was observed to be reduced by 26 to 69 percent due to dye treatment. A reduction of house fly fecundity was observed to be directly related to the dietary concentration of the rose bengal and the frequency of feeding.

Even though there was no remarkable effect on the viability of the eggs, there seemed to be approximately a 5 to 26 percent reduction in the viability of eggs laid by the female house flies which were fed on rose bengal (92). There was no significant change in

the sex ratio due to the photosensitizers in mosquitoes (73) and
house flies (Pimprikar, unpublished)

Barenbaum and Freeny (77) while studying the effect of the
furanocoumarins in Papilio, observed that there was a 3- to 5-fold
difference in the average egg production between the control and
angelicin treatments. The individual butterflies in the control
treatments laid up to 700 more eggs than the treated females. The
authors correlated the reduced pupal weights with the reduced body
size which also correlates with fecundity. The assumption here is
that the insects which are developed on the medium treated with the
photosensitizer produce abnormally smaller and lighter weight indi-
viduals and these adults are not capable of producint a normal
complement of eggs.

It has been observed that all the life stages of insects are
susceptible to the action of photosensitizers and these compounds
are capable of causing toxicity from the egg to the adult stage.
When house fly eggs are treated with photosensitizers and exposed
to light, several of the xanthene dyes exhibited ovicidal activity
(92). The relatively flat slopes of the log dose versus probit
mortality lines indicate that the rates of penetration of the pho-
tosensitizers through the chorion is very slow or the eggs are not
as sensitive to dyes as the other life stages. Some of the treated
house fly eggs totally fail to hatch probably due to the death of
the embryo. In some cases, the larvae free themselves from the
head capsule, but the caudal end still remains in the egg shell.
Various other abnormalities in the hatching of the eggs were
observed. Eosin Y and Phloxin B treatment caused pitting of egg
cell membranes, vacuole formation, and eventual disintegration in
sea urchin eggs (93). Kagan and Chan (94) reported the ovicidal
effects of some of the photodynamic natural products in D. melano-
gaster and suggested that the photosensitized enhancement of the
ovicidal activity can be appreciably increased by properly
selecting the irradiation period.

Quantitative studies on the effects of photosensitizer treat-
ment at various larval and pupal stages on adult emergence were
conducted in face flies (76) and house flies (92). Figure 3 sum-
marizes the effects of the rose bengal treatment at each stage of
development in the house fly.

The dye treated female house flies produce relatively fewer
eggs and these eggs are comparatively less viable. The dye treated
eggs show ovicidal activity resulting in reduction in the egg
hatch. The larvae reared on the medium containing the photosen-
sitizers exhibit increased mortality prior to pupation and resulted
in up to 80 percent reduction in adult emergence depending on the
stage of exposure and the concentration of the photosensitizer
(Fig. 3).
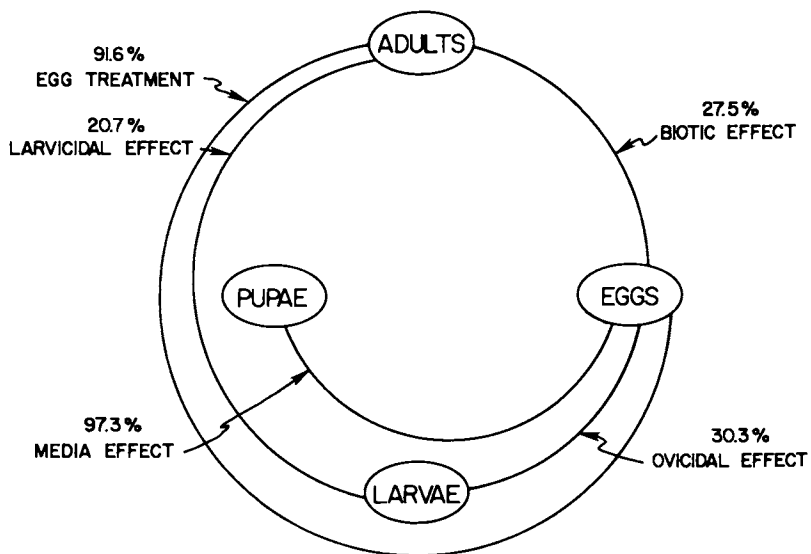
Figure 3.   The developmental effects of rose bengal on various stages of house fly.

These studies add to the concept that the photosensitizers are capable of causing toxicity from the egg to the adult stage and that the effects are complex. It is very difficult to analyze the effects at each stage which also suggests that the eventual field effectiveness would be difficult to estimate based on a study of toxicity at a single life stage.

In conclusion, there are several toxic mechanisms in operation at a given time in addition to the light dependent toxic mechanism. It is very difficult to isolate or define the relative contribution of each of these mechanisms at a given time.

Acknowledgments

Literature Cited

1. Bakker, J.; Gommers, F.J.; Nieuwenhuis, I.; Wynberg, H. J. Biol. Chem. 1979, 254, 1841-1844.
2. Lochmann, E.R.; Micheler, A. In Physico-chemical Properties of Nucleic Acids; Ducheswe, J. Ed.; Academic Press, New York, 1973;1, Chapter 8.
3. Spikes, J.D.; Livingston, R. Adv. Radiat. Biol. 1969, 3, 29-121.
4. Spikes, J.D. In The Science of Photobiology; Smith, K.C., Ed.; Plenum, New York, 1977; 87-112.
5. Foot, C.S. In Free Radicals in Biology; Pryor, W.A., Ed.; Academic, New York, 1976; 85-133.
6. Robinson, J.R.; Beatson, E.P. Pest. Biochem. Physiol. 1985, 24, 375-383.
7. Amagasa, J. Photochem. Photobiol. 1981, 33, 947-955.
8. Bezman, S.A.; Burtis, P.A.; Izud, T.P.J.; Thayer, M.A. Photochem. Photobiol. 1978, 28, 325-329.
9. Miyushi, N.; Tomita, G. Photochem. Photobiol. 1979, 29, 527-530.
10. Usui, Y. In Singlet oxygen; Rawby, B.; Rabek, J.F., Eds.; Wiley, Chichester, England, 1978; 203-210.
11. Nieumint, A.W.M.; Aubry, J.M.; Arwert, C.; Kortbeck, H.; Herzberg, S.; Joenje, H. Free Radical Res. Communications, 1985, 1, 9.
12. Glazer, A.N. Proc. Natl. Acad. Sci. U.S. 1970, 65, 1057-1063.
13. Straight, R.C.; Spikes, J.D. In Singlet oxygen; Frimer, A.A., Ed.; CRC, Boca Raton, Florida; 1985; 4, 91-143.
14. Jori, G. ; Spikes, J.D. In Oxygen and Oxy-Radicals in Chemistry and Biology; Rodgers, M.A.J.; Powers, E.L., Eds.; Academic Press, New York, 1981; 441-459.
15. Lamola, A.A.; Yamane, T.; Trozzolo, A.M. Science, 1973, 179, 1131-1133.
16. Ito, T. Photochem. Photobiol. 1978, 28, 493-508.

17. Song, P.S.; Tapley, K.J., Jr. Photochem. Photobiol. 1979, 29, 1177-1197.
18. Bellin, J.S.; Grossman, L.C. Photochem. Photobiol. 1965, 4, 45-53.
19. Philogene, B.J.R.; Arnason, J.T.; Duval, F. Can. Entomol. 1985, 117, 1153-1157.
20. Arnason, J.T.; Fortier, G.; Champagne, D.; Philogene, B.J.R. Rev. Can. Biol. Exp. 1983, 42, 205-208.
21. Pathak, M.A. ; Kramer, D.M.; Fitzpatrik, T.B. In Sunlight and Man; Pathak, M.A.; Harber, L.C.; Seiji, M.; Kukita,A.; Eds.; University of Tokyo Press, Tokyo, 1974; pp.335.
22. Wages, J. Ph.D. Dissertation Mississippi State University, Mississippi State, 1985.
23. Miller, A.C.; Henderson, B.W. Radial. Res. 1986, 107, 83-94.
24. Yoho, T.P. Ph.D. Dissertation, West Virginia University, Morgantown, 1972.
25. Barbieri, A. Riv. Malariol. 1928, 7, 456-463.
26. Weaver, J.E.; Butler, L.; Yoho, T.P. Environ. Entomol. 1976, 5, 840-844.
27. Yu, B.P.; Masuro, E.J.; Bertrand, H.A. Biochem. 1974, 13, 5083-5087.
28. Takahara, J.; Yunoki, S.: Yamauchi, J.; Yakushiji, W.; Hashimoto, K; Ofuji, T. Life Science, 1981, 29, 1229-1233.
29. Duncan, C.J.; Bowler, J. J. Cell Physiol. 1970, 79, 259-271.
30. MacRae, W.D.; Irwin, D.A.; Bisalputra, T.; Towers, G.H.N. Photochem. Photobiophys. 1980, 1, 309-318.
31. Downum, K.R.; Hancock, R.E.W.; Towers, G.H.N. Photochem. Photobiol. 1982, 36, 517-523.
32. Freeman, P.J.; Giese, A.C. J. Cellular Comp. Physiol. 1952, 39, 301-322.
33. Pooler, J.P.; Valenzeno, D.P. Biochem. Biophys. Acta, 1979, 555, 307-315.
34. Allison, A.C.; Magnus, I.A.; Young, M.R. Nature, 1966, 209, 874-878.
35. Cande, W.Z.; McDonald, K.; Meeusen, R.L. J. Cell Biol. 1981, 88, 618-629.
36. Haga, J.Y.; Spikes, J.D. In Organic Biological and Medicinal Chemistry; Galo, U.; Santamaria, L., Eds.; American Elsevier Publishing Co., New York, 1972; 3, 464-479.
37. Daub, M.E.; Briggs, S.P. Plant Physiol. 1983, 71, 763-766.
38. Wagner, S.; Taylor, W.D.; Keith, A.; Snipes, W. Photochem. Photobiol. 1980, 32, 771-779.
39. Ito, T. Photochem. Photobiol. 1980, 31, 565-570.
40. Tudball, N.; Thomas, P. Biochem. J. 1971, 123, 421-426.
41. Brand, K.; Tsolas, O.; Horecker, B.L. Arch. Biochem. Biophys. 1969, 130, 521-529.
42. Rosenthal, I. Photochem. Photobiol. 1976, 24, 641-645.
43. Rahimtula, A.D.; Hawco, F.J.; O'Brien, P.J. Photochem. Photobiol. 1978, 28, 811-815.
44. Carraro, C.; Pathak, M.A.; Bissett, D.L. Photochem. Photobiol. 1986, 43, 14S.
45. Tsai, C.S.; Godin, J.: Wand, A.J. Biochem. J. 1985, 225, 203-208.

46. Garcia, F.J.; Yamamoto, E.; Abramowski, Z.; Downum, K.;
     Towers, G.H.N. Photochem. Photobiol. 1984, 39, 521-524.
47. Knox, J.P.; Dodge, A.D. Planta, 1985, 164, 22-29.
48. Kaye, N.M.C.; Weitzman, P.D.J. FEBS Lett. 1976, 62, 334-337.
49. Fu., N.; Yeh, S.; Chang, C.; Zhao, X.; Chang, L. Adv. Exp.
     Med. Biol. 1985, 193, 161-167.
50. Gommers, F.J.; Bakker, J.; Smits, L. Nematologica, 1980, 26,
     369-375.
51. Callaham, M.F.; Lewis, L.A.; Holloman, M.E.; Broome, J.R.
     Heitz, J.R. Comp. Biochem. Physiol. 1975, 51C, 123-128.
52. Callaham, M.F.; Palmertree, C.O.; Broome, J.R.; Heitz, J.R.
     Pest. Biochem. Physiol. 1977, 7, 21-27.
53. Broome, J.R.; Callaham, M.F.; Poe, W.E.; Heitz, J.R. Chem.
     Biol. Interact. 1976, 14, 203-206.
54. Callaham, M.F.: Broome, J.R.; Poe, W.E.; Heitz, J.R. Environ.
     Entomol. 1977, 6, 669-673.
55. Blum, H.F. J. Invest. Dermatol. 1941, 4, 159-173.
56. Broome, J.R.; Callaham, M.F.; Louis, L.A.; Ladner, C.M.;and
     Heitz, J.R. Comp. Biochem. Physiol. 1975, C51, 117-121.
57. Fondren, J.E., Jr.; and Heitz, J.R. Environ. Entomol. 1978, 7,
     843-846.
58. Fondren, J.E., Jr.; Heitz, J.R. Environ. Entomol. 1979, 8,
     432-436.
59. Creighton, C.S.; McFadden, T.L.; and Schalk, J.M. J. Ga.
     Entomol. Soc. 1980, 15, 66-68.
60. Carpenter, T.L.: Hetiz, J.R. Environ. Entomol. 1981, 10,
     972-976.
61. Respicio, N.C.; Heitz, J.R. Bull. Environ. Contam. Toxicol.
     1981, 27, 274-281.
62. Wat, C.K.; Prasad, S.K.; Graham, E.A.; Partington, S; Arnason,
     T.; Towers, G.H.N. Biochem. Syst. and Ecol. 1981, 9, 59-62.
63. Arnason, T.; Swain, T.; Wat, C.K.; Graham, E.A.; Partington,
     S.; Towers, G.H.N.; Lam, J. Biochem. Syst. and Ecol. 1981, 9,
     63-68.
64. Berenbaum, M. Science, 1978, 201, 532-534.
65. Champagne, D.E.; Arnason, J.T.; Philogene, B.J.R.; Campbell,
     G.; Malachlan, D.G. Experientia, 1984, 40, 577-578.
66. Yamamoto, E.; Wat, C.K.; MacRae, W.D.; Towers, G.H.N.; Chan,
     G.F.Q. FEBS Lett. 1979, 107, 134-136.
67. Tauton, M.T.; Khan, S.M. Aust. J. Zool. 1978, 26, 139-146.
68. Waldbauer, G.P. Adv. Insect. Physiol. 1968, 5, 229-288.
69. Champagne, D.E.; Arnason, J.T.; Philogen, B.J.R.; Morand, R.;
     Lam, J. J. Chem. Ecol. 1986, 12, 835-858.
70. Jordan, T.W.; Smith, J.N. Xenobiotica, 1981, 11, 1-7.
71. David, J. Bull. Biol. France Belgique, 1963, 97, 515-530.
72. Kolyer, J.M. J. Res. Lep. 1966, 5, 136-152.
73. Barbosa, P.; and Peters, T.M. Entomol. Exp. Appl. 1970, 13,
     293-299.
74. Bridges, A.C.; Cocke, J.; Olson, J.K.; Mayer, R.T. Mosquito
     News. 1977, 37, 227.
75. Pimprikar, G.D.; Norment, B.R.; and Heitz, J.R. Environ.
     Entomol. 1979, 8, 856-859.
76. Fairbrother, T.E. Ph.D. Dissertation, Mississippi State
     University, Mississippi State, 1978.
77. Berenbaum, M.; Feeny, P. Science, 1981, 212, 927-929.

78. Downum, K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest. Biochem.
     Physiol. 1984, 22, 104-109.
79. Fairbrother, T.E.; Essig, H.W.; Combs, R.L.; Heitz, J.R.
     Environ. Entomol. 1981, 10, 506-510.
80. Rehr, S.S.; Janzen, D.H.; Feeny, P.P. Science, 1973, 181,
     81-82.
81. Hsiao, T.H.; Hsiao, C. J. Insect Physiol. 1977, 23, 89-93.
82. Pimprikar, G.D.; Coign, M.J.; Sakurai, H.; Heitz, J.R. J.
     Chrom. 1984, 317, 413-419.
83. Edwards, W.F. Text. World. 1921, 60, 1111-1113.
84. Barbosa, P.; Peters, T.M. J. Med. Entomol. 1970, 7, 693-696.
85. Clement, S.L.; Schmidt, R.S.; Szatmari-Goodman, G.; and
     Levine, E. J. Econ. Entomol. 1980, 73, 390-392.
86. Kagan, J.; Hasson, M.; Grynspan, F. Biochim. Biophys. Acta,
     1984, 802, 442-447.
87. McLachlan, D.; Arnason, J.T.; Philogene, B.J.R.; Champagne, D.
     Experientia, 1982, 38, 1061-1062.
88. Jermy, T.; Butts, B.A.; McDonough, L. Insect Sci. Appl. 1981,
     1, 237-242.
89. Sakurai, H.; Heitz, J.R. Environ. Entomol. 1982, 11, 467-470.
90. Lewontin, R.C. In The Genetics of Colonizing Species; Baker,
     H.G.; Stebbins, G.L., Eds.; Academic Press, New York, 1965;
     pp.588.
91. David, J. C.R. Acad. Sci. Paris. 1955, 241, 116-118.
92. Pimprikar, G.D.; Noe, B.L.; Norment, B.R.; Heitz, J.R.
     Environ. Entomol. 1980, 9, 785-788.
93. Tennent, D.H. In Papers From Tortugas Laboratory Vol. XXXY;
     Carnegie Institute of Washington, Washington, D.C. 1942;
     Publication #539.
94. Kagan, J.; Chan, G. Experientia, 1983, 39, 402-403.

Chapter 10

Field Development of Photooxidative Dyes as Insecticides

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Erythrosin B (Synerid) a photooxidative
dye has been shown to have insecticidal
properties against adult house flies in
small scale poultry tests conducted in FL.
It provided up to 95% reduction of the adult
house fly population in one of these tests.
It is not, however, commercially satisfac-
tory as a house fly larvicide. Erythrosin
B, acridine red, and rose bengal have all
been used experimentally to control mosquito
larvae in small pools. Erythrosin B also
shows promise as a single mound treatment
for control of red imported fire ants
(RIFA). It controlled RIFA colonies as
effectively as Amdro through 56 day post-
treatment. The photooxidative dyes are
extremely safe to man and the environment.
Erythrosin B has an LD_{50} of 6,000-7,000
mg/kg of body weight.

Since this is a symposium on light-activated pesticides,
it only seems right that the field evaluation and
commercial development of these compounds be examined.
The true test for any pesticide is how it performs under
actual field conditions.
     Various studies have shown that a number of insect
species exhibit photooxidative toxic reactions when
exposed to certain dyes. Dyes such as erythrosin B and
rose bengal are effective control agents against the
adult stage of the house fly (1-4), face fly (5), black
imported fire ant (6), and boll weevil (7-8). Toxic
reactions to these dyes in the larval stage of
mosquitoes (9-11), house and face flies (12-14), yellow
mealworms (15), cabbage butterflies (16), and black
cutworms (17) have been observed in the laboratory.

     Despite the promising indications of these early
laboratory experiments, little work has been conducted
to evaluate the insecticidal activity of the dyes in the
field. Most of the field work so far involved tests in
poultry facilities for the control of house flies using
erythrosin B under the name Synerid Fly Control B. At
present, this is the only dye registered and
commercially available for insect control. This product
is labelled for control of house flies in confined
animal areas, including poultry facilities.
     One of the greatest advantages of photooxidative
dyes is their low mammalian toxicity (Table I).
Erythrosin B is relatively harmless to mammals (18-19)
and has an acute oral LD_{50} of 6,700-7,000 mg/kg of body
weight in rats (19). A number of other dyes also show
low mammalian toxicity when compared to commonly used
insecticides (Table I) (20-21). Besides their low
mammalian toxicity, these compounds degrade rapidly in
the environment (22) and there is little threat of
contaminating water sources or accumulating in the food
chain. The photooxidative dyes are extremely safe and
pose no threat to the health or welfare of the
applicator or environment in field usage.

            Table I.   The LD_{50} for Various Dyes Which Show
                       Insecticidal Properties.[a]

Compound	Acute Oral[1]	Intravenous[1]
Artic white Tx	16,000*	–
D&C red #22	2,344	550
FD&C blue #02	–	93*
FD&C red #03	1,264	700
FD&C yellow #06	12,750	–
Methylene blue	1,180*	82
Sodium fluorescein	6,721*	–
Erythrosin B	6,700*	–
Eosin yellow	2,340	–

[a]RTECS, Niosha, Supt. of Documents, U.S. Government
  Print Office: Washington, DC, 1983.
[1]This figure represents mg/kg of body weight
  (for mice) required to kill 50% of the test
  population.
*Rats were used as test organism.

House Fly Field Experiments

Larvicide Tests.   Field tests were necessary to satisfy
EPA requirements for registration. Poultry facilities
offer a perfect environment for testing products against
house flies since they are ideal fly-breeding habitats.

This is because in most caged layer operations the
manure is allowed to accumulate under caged hens
providing excellent oviposition and larval development
sites (23). Unchecked fly development results in adult
flies creating a nuisance.
     Since house flies are developing resistance to
many of the currently used products like the synthetic
pyrethroids and cyromazine (24-25), the poultry
industry needs alternatives for house fly control. The
new, safer photooxidative dyes may fill this
requirement.
     One of the earliest field tests using Synerid was
conducted at the Mississippi State University to control
house flies in large and small scale field tests (26).
In both studies, manure was sprayed weekly with an
aqueous solution of erythrosin B for 4 weeks. Manure
samples were collected in order to assess larval
populations, while sticky traps were used to assess
adult house fly populations.
     House fly populations were reduced up to 94% and
89% in the small and large scale tests, respectively.
The data also indicated that larval densities of the
associated beneficial soldier fly, Hermetia illucens,
were not reduced. This is an important observation
since the most desirable control agents are those that
do not negatively affect beneficial insects while
simultaneously controlling pest populations. Further
analysis of the manure during this study indicated that
erythrosin B rapidly degraded under the encountered
environmental conditions. This feature alleviates
concerns about pesticide residues in chicken manure
which is often used as fertilizer.
     Following this study, erythrosin B was registered
by Sterling Drug Inc. as Intercept to be marketed as a
larvicide for house fly control in caged layer
facilities. Later, this compound was reregistered as
Synerid by Hilton Davis.
     After EPA registration of the dye, Hilton Davis
Chemical Company conducted further field tests of the
product for larval fly control in California, South
Carolina, Indiana and Florida. Results of these
studies, with one exception, remain unpublished.
     The California study evaluated Synerid and Synerid
100 (70% erythrosin B and 30% sodium fluorescein) in
both small and large plots (27-28). In small field
tests, three rates of Synerid and Synerid 100 were
tested. Only one rate of each product was used in the
large scale field tests. In both tests, the materials
were applied on a weekly basis.
     Synerid 100 treatments resulted in significantly
fewer larvae and adults relative to the controls. Adult
fly populations in Synerid treated houses, however, did
not differ significantly from either Synerid 100 or the
control. The large scale test also indicated that

Synerid 100 significantly reduced house fly larval
densities relative to control samples from another
house. Despite these significant reductions the level
of fly control was commercially unsatisfactory.
      A second study conducted in California, also,
showed that weekly spraying of the manure with labelled
rates of Synerid did not control house fly larvae (29).
Adult house fly populations, monitored with sticky
tapes, were never below pretreatment levels in both the
control and Synerid treated houses.
      Similar results were obtained in large scale field
tests conducted in South Carolina (Nolan, III, M. P.,
Clemson University, personal communication) and in a
small field test at the USDA-ARS laboratory in
Gainesville, Florida.
      The preliminary field test in Florida was conducted
in four outdoor fly-proof screened rooms (2.43 X 4.88 m)
in 1985. Each room was provisioned with a rack of 13
cages (.2 X .45 X .41 m) each holding two White Leghorn
layers. Manure was allowed to accumulate under the
cages and wild adult house flies were allowed access to
the rooms through the doors during the care of the
birds. Weekly treatments of erythrosin B were initiated
and one of four treatments was randomly assigned (using
a random numbers table) to each room. Treatments were
as follows: 139.5 mg, 209.3 mg, 294.0 mg/ 62.00 ml
water/ m^2, and no treatment-control. Population
assessments were conducted by counting the number of
adult house flies caught on one sticky tape in a 24 hour
period. The tape was hung under a randomly chosen cage
in each room. Only one tape was used per room to insure
that the house fly population was not eliminated through
its capture on the tape.
      Table II indicates that the total number of adult
flies caught by the sticky tape in each room did not
decrease from pretreatment levels after the application
of erthyrosin B. Since only one tape was used in each
room and the experiment was not replicated the results
were inconclusive. However, communication with
researchers conducting similar studies in California,
South Carolina, and Indiana strongly suggested that
these results were typical. Therefore, it seemed
pointless to replicate such a labor intensive study
until more was known about the biological properties of
the product.
      The slight decline in population levels that was
seen at the end of the study was most likely the result
of parasitism and predation by beneficial arthropods.
Ninety percent of the pupae returned to the laboratory
to monitor for fly emergence were parasitized by
Muscidifurax raptor, a common house fly parasite. This
observation corroborates the earlier study (26) which
found erythrosin B had little to no effect on beneficial
arthropods when the material was sprayed on the manure.

During this study, however, adult house flies with
red abdomens were found resting on the walls of the room
following the spraying of manure with erythrosin B. We
assumed that these flies were produced from treated
larvae, which appeared pink in color, and, would
therefore ultimately die. However, none of these flies
brought into the laboratory died. Instead the abdomens
of all the flies returned to their normal color within
24 h; the flies continued to live and red fecal and oral
spots were observed in the cages. This indicated that
flies were able to successfully clean their abdomens of
the dye.

Table II.   Total Number of House Fly Adults Trapped
            on a Sticky Tape Following the Spraying of
            the Manure with 1 of 3 Rates of Erythrosin
            B in a Small-Scale Field Test.
            (Gainesville, Florida 1985)

Julian Date	Control	Treatment[a] Low	Medium	High
102	161	55	16	37
109	600	650	575	830
116	275	450	500	650
123	550	450	225	500
130	150	85	200	300
137	400	200	175	225
144	250	175	225	200
152	175	110	175	150

[a] Treatments were applied on Julian date 102, 1985.
    Rates were: low=139.5 mg, medium=209.3 mg, and high=
    279.0 mg/62.00 ml water/m^2.

It was later noted that prior to spraying the
manure no red-abdomened flies were observed. However,
within 4 h of spraying the manure, approximately 60% of
the adult house flies had red abdomens. From these
observations it was concluded that: 1) red flies were
not the result of their feeding on erythrosin B during
their larval stage, 2) adult house flies would feed on
the erythrosin B spray while it was wet, and 3) some of
the adults that did ingest the dye were able to
eliminate it through defecation and regurgitation.

Adulticide Tests. The observations made in the Florida
field test indicated that Synerid may in fact be an
effective adulticide when fed to adult flies in large
enough amounts. Therefore, erythrosin B was returned to
the laboratory for a closer evaluation of its
adulticidal activity. Research from these laboratory

studies indicated that erythrosin B (Synerid) showed more promise as an adulticide than a larvicide (4).

Following the laboratory studies, small scale field evaluations of erythrosin B as a liquid adult bait were conducted in 1985 and 1986. The purpose of the field testing was two-fold: 1) to evaluate control of adult house flies with erythrosin B and 2) to observe rates of fly reduction when muscalure was used in conjunction with the dye. Laboratory experiments indicated that certain lethal concentrations of erythrosin B inhibited ingestion by house flies. Therefore, it seemed necessary to keep the flies at the bait station so that adult flies would ingest enough material to ensure death. Muscalure was used since it acts as a feeding arrestant for house flies (30-31). The results of these studies have not yet been published, therefore, the materials and methods are also described.

Three of the four fly-proof screened rooms used in the previously described small scale field test were utilized in this experiment. Chick watering towers were used as the bait stations. Each device had a circular trough containing dye solutions. Sterile cotton was provided as a support medium so that flies could rest on it and ingest the fluids. All bait stations were placed on the ground in the southwest corner of each room containing the chicken cages. Solutions in the watering towers were replaced weekly. One of three treatments (Synerid, Synerid + muscalure and a control) was placed in each room.

Adult house fly populations in all rooms were sampled daily for five days preceding treatment with a modified Scudder grid. From the day the treatments were applied, sampling was done daily for 3 weeks post-treatment except on weekends. The sampling procedure used a modified 44 cm square Scudder grid consisting of 12 strips of wood 44 cm long and 2 cm wide spaced 2 cm apart. The grid was randomly placed on the ground in each room for 30 seconds and the number of flies which rested on the grid after the 30 second interval were counted. Grid counts were replicated 5 times in each room.

Results indicated that application of Synerid (1% by volume erythrosin B bait) with muscalure resulted in an average 95% and 51% reduction of house flies from pretreatment levels in studies 1 and 2, respectively (Table III and IV). Synerid without muscalure resulted in an average 67% reduction of the initial house fly population in study 1 and 34% in study 2. In control rooms house fly populations increased from pretreatment levels by an average 10% in study 1 and 31% in study 2. At present this brings the field research on erythrosin B for house fly control up-to-date. Besides the field evaluations of erythrosin B for fly control, a little field work has been conducted at examining the

feasibility of using these dyes to control other insect species.

Table III. Percent Reduction of House Fly Populations Following Application of Synerid in a Small Scale Poultry Facility (Florida, 1985).

Day posttreatment	Percent Reduction[a]		
	Control	Synerid	Synerid+muscalure
2	−35.36	60.58	79.45
4	19.51	56.20	95.89
8	−28.04	71.53	87.67
10	−50.00	64.96	95.89
14	52.43	34.03	91.78
18	15.85	80.29	91.78
22	−60.97	73.77	100.00
Average	−10.00	67.00	95.87

[a] When percent reduction is preceded by a minus sign this represents an increase by that percent in the population from the pretreatment level.

Table IV. Percent Reduction of House Fly Populations Following Application of Synerid in a Small Scale Poultry Facility (Florida, 1986).

Days posttreatment	Percent Reduction[a]		
	Control	Synerid	Synerid+muscalure
2	54.50	15.06	64.60
4	89.16	27.38	70.28
8	79.45	53.30	80.50
10	−16.96	8.02	78.08
14	−235.74	−23.28	30.66
18	−10.10	54.33	58.72
22	−58.12	41.09	51.88
Average	−31.48	34.60	51.26

[a] When percent reduction is preceded by a minus sign this represents an increase by that percent in the population from the pretreatment level.

Mosquito Field Tests

Two small scale field studies have evaluated the use of fluorescent dyes and erythrosin B for control of mosquito larvae. In the first study which was undertaken in Germany, results indicated that acridine red and rose bengal showed efficacy in dilutions of 1:100,000 (32). Exposure of Anopheles larvae to acridine red in 6 of 10 treated small pools resulted in 90 to 100% mortality. In the other 4 pools the results

were variable (32). The indication was that these two
dyes may be safe and effective mosquito control agents.
     Recently Carpenter et al. (33) evaluated erythrosin
B for mosquito control. This experiment was conducted
in 30 1 x 1 x 0.05m holes that had been dug in the
ground. Each hole was lined with plastic sheets and
then filled with well water for a 25 cm depth. Prior to
initiation of the experiment 500 to 600 fourth instar
Culex pipiens quinquefasciatus larvae were transferred
into each test plot. Larval samples were taken using a
dipper 24 h after the application of the erythrosin B.
The results showed that at 8.0 ppm 96% and 92%
reductions in larval populations could be seen in
studies 2 and 3, respectively. Fifty percent control
could be seen in 24 h at treatment rates of 0.5 to 1.0
ppm (33). The results of study 1 indicated that
effective applications of erythrosin B for mosquito
control was dependent on the pH of the water to be
treated. The pH of the water is an important factor for
effective control of mosquito larvae when using dyes.

Fire Ant Field Experiments

Two studies have also been conducted to assess the
toxicity of certain dyes on field colonies of imported
fire ants. In the first study, field collected mounds
of Solenopsis richteri, the black imported fire ant,
were dug up and brought back to the laboratory where
they were maintained (34). Colonies were then fed
soybean oil baits which contained the dye phloxin B. It
was found that phloxin B caused mortality to colonies
and the amount of time it took for death to occur was
dependent on the amount of light to which they were
exposed. Since these were field collected colonies, it
can be hypothesized that similar results would be
obtained under actual field conditions. However, it
would be necessary to see if this particular bait is as
attractive to foraging ants. If the queen does not
ingest the bait and die then colony life will go on
undisturbed. Therefore, it is essential to study the
reactions of foraging fire ants in the field to the
material, before extrapolating laboratory observations
to field situations.
     A large scale field test was conducted by the
author in order to assess the efficacy of a number of
baits for control of individual red imported fire ant
(RIFA), S. invicta, colonies (35). Included in this
evaluation was a soybean oil bait which contained
erythrosin B (supplied by the Hilton Davis Co.,
Cincinnati, OH). The control of erythrosin B treated
colonies was not significantly different (P>0.05) than
that of colonies treated with standard commercial bait
Amdro (Table V) for 56 days post-treatment. The degree
of control with all baits, however, was not

Table V. Field Evaluation of Baits for Use as Single Mound Treatments for Control of Red Imported Fire Ants

Bait	No. Mounds Treated	Days Post-Treatment*				
		21	42	56	84	112
Amdro	31	71.2 a**	55.1 abc	53.1 a	50.3 bc	44.3 c
Affirm A003	30	46.4 a	67.1 ab	66.9 a	36.3 c	38.1 c
Affirm W002	30	59.6 a	85.4 a	75.2 a	83.4 a	78.3 a
Pro-Drone (31.0g)	33	15.7 b	22.0 ed	40.4 a	53.5 bc	53.3 bc
Pro-Drone (12.4g)	30	14.0 b	26.8 cd	48.5 a	73.8 ab	73.8 ab
Synerid	30	53.3 a	48.2 bc	43.8 a	7.8 d	8.7 d
Control	31	0.0 b	0.0 d	0.0 d	0.0 d	0.0 d

*Treatments were made 4 June 1984
** Values expressed as percent of treated mounds that showed inactivity.
Means within the same column, followed by the same letter, are not significantly different (P> 0.05) according to the Least Squares Difference test.

satisfactory. Additional field tests need to be
conducted before recommending this product for RIFA.

Summary

Although, there are many references to the biological
activity of photoactive dyes on insects in the
literature, little of it addresses the effectiveness of
them in the field. It is important to remember that
positive results in the laboratory does not assure its
success in the field. Many elements such as weather,
sunlight, humidity, and pH can cause products to be
ineffective. The real test for these dyes lies in
additional field tests and it is hoped that more field
oriented studies will be attempted. Commercial
development has already shown that such a product
(Synerid) stands a good chance in the market place if
reliable information can be obtained in the field.
Laboratory developers and field researchers must work
together in order to assure the success of these
products. Indeed they are a most attractive group of
insecticides when one considers the safety and
selectivity of these compounds.
      The development of these products is justified
since they are extremely safe with many of them being
registered as food additives. Due to this safety, there
is low cost for toxicological testing in order to
satisfy EPA requirements. Because little toxicological
testing is needed, speedy registration of the product by
EPA can be anticipated. In addition, few label
restrictions are required since there is little hazard
to the applicator, crops, domestic animals, wildlife or
fish. In an age of health and environmentally
conscience individuals, these products can be used
without controversy.

Literature Cited

1.   Yoho, T. P.; Butler, L.; Weaver, J. J. Econ.
     Entomol. 1971, 64, 972-3.
2.   Yoho, T. P.; Weaver, J. E.; Butler, L. Environ.
     Entomol. 1973, 2, 1092-6.
3.   Yoho, T. P.; Butler, L.; Weaver, J. E. Environ.
     Entomol. 1976, 5, 203-4.
4.   Koehler, P. G.; Patterson, R. S. J. Econ. Entomol.
     1986, 79, 1023-26.
5.   Fondren, Jr., J. E.; Heitz, J. R. Environ.
     Entomol. 1978, 7, 843-6.
6.   Broome, J. R.; Callaham, M. F.; Lewis, L. A.;
     Ladner, M. C.; Heitz, J. R. Comp. Biochem.
     Physiol. 1975, 51, 117-21.
7.   Callaham, M. F.; Broom, J. R.; Lindig, O. H.;
     Heitz, J. R. Environ. Entomol. 1975, 4, 837-41.

8.   Broome, J. R.; Callaham, M. F.; Poe, N. R.; Heitz,
     J. R. Chem.-Biol. Interact. 1976, 14, 203-6.
9.   Barhieri, A. Rivista di Malariologica 1928, 7,
     456-63.
10. Barbosa, P.; Peters, T. M. Mosq. News 1969, 29,
     243-51.
11. Pimprikar, G. D.; Norment, Jr., B. R.; Heitz, J. R.
     Environ. Entomol. 1979, 8, 856-9.
12. Fairbrother, T. E. Ph.D. Thesis, Mississippi State
     University, Starkville, 1978.
13. Sakurai, H.; Heitz, J. R. Environ. Entomol. 1982,
     11, 467-70.
14. Pimprikar, G. D.; Noe, B. L.; Norment, B. R.;
     Heitz, J. R. J. Econ. Entomol. 1980, 73, 785-8.
15. Graham, K.; Wranger, E.; Sasan, L. H. Can. J.
     Zool. 1972, 50, 1625-9.
16. Lavialle, M.; Dumortier, B. C. R. Acad. Sc. Paris
     1978, 287, 875-8.
17. Clement, S. L.; Schmidt, R. S.; Szatmari-Goodman,
     G.; Heitz, J. R. J. Econ. Entomol. 1980, 73,
     390-392.
18. Lu, F. C.; Lavalle, A. Can. Pharm. J. 1964, 30,
     530.
19. Butterworth, K. R.; Gaunt, I. F.; Grasso, P.;
     Gangolli, S.D. Fd. Cosmet. Toxicol. 1976, 14,
     525-31.
20. RTECS, NIOSHA, Supt. of Documents, U.S. Government
     Print Office: Washington, DC, 1983.
21. Farm Chemicals Handbook, 1977, 5th ed.
22. Heitz, J. R. Disposal and Decontamination of
     Pesticides; Kennedy, M. V., American Chemical
     Society: Washington, DC, 1978; No. 73, p 35-48.
23. Axtell, R. C.; Rutz, D. A. Entomol Soc. Am. Misc.
     Publ. 1986, 61, 88-100.
24. Hinkle, N. C.; Sheppard, D. C.; Nolan, Jr., M. P.
     J. Econ. Entomol. 1985, 78, 722-4.
25. Bloomcamp, L. M.S. Thesis, University of Florida,
     Gainesville, 1986.
26. Pimprikar, G. D., Fondren, Jr., J. E.; Heitz, J. R.
     Environ. Entomol. 1980, 9, 53-8.
27. Meyer, J. A.; Mullens, B. A.; Rooney, W. F.;
     Rodriguez, J. L. J. Agric. Entomol. 1986, 2,
     351-7.
28. Meyer, J. A.; Mullens, B. A.; Rooney, W. F.
     Progress in Poultry; Univ. California Coop.
     Extension Serv., 1986, No. 31, 6 pp.
29. Meyer, J. A.; Bradley, F. Progress in Poultry;
     Univ. California Coop. Extension Serv., 1986,
     No. 34, 3 pp.
30. Carlson, D. A.; Mayer, M. S.; Silhacek, D. L.;
     James, J. D.; Beroza, M.; Bierl, B. A. Science
     1971, 174, 76-8.
31. Carlson, D. A.; Beroza, M. Environ. Entomol.
     1973, 2, 555-9.

32. Schildmacher, H. Biol. Zentr. 1950, 69, 468-77.
33. Carpenter, T. L.; Respicio, N. C.; Heitz, J. R. J. Econ. Entomol. 1985, 78, 232-7.
34. David, R. M.; Heitz, J. R. J. Agric. Food Chem. 1978, 26, 99-101.
35. Lemke, L. A. Ph.D Thesis, Clemson University, Clemson, SC, 1986.

Chapter 11

Photodecomposition of Naturally Occurring Biocides

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Light-activated biocides include a wide range of naturally
occurring compounds from plants as well as some synthetic dye
molecules. These chemicals are active against microorganisms,
insects and nematodes, as well as snails and fish in vitro.
Many are potentially useful as commercial pesticides, a
property particularly enhanced by evidence of rapid
biodegradability in the environment. The mechanisms of
photodegradation and the factors which influence this process
will be discussed in this review.

Many kinds of compounds have been reported to be toxic to biological
systems in the presence of light under aerobic and anaerobic
conditions. In photodynamic reactions the photon-excited sensitizer
molecule transfers its excitation energy to oxygen, generating the
singlet state which may subsequently react with phospholipids,
proteins and sterols of cellular membranes. A structurally diverse
group of phytochemicals isolated from plants has been reported to
exhibit biocidal activity towards viruses, bacteria, fungi,
nematodes and insects in the presence of sunlight or UV-A radiation
(320-400nm) (1-4). Such compounds include various alkaloids (5,6),
acetophenones (7), extended anthraquinones (4), furanocoumarins
(8,9), furochromones (10), straight-chain and aromatic polyacetyl-
enes, and thiophenes (e.g. 11-15). In addition, synthetic xanthene
dyes such as rose bengal are well known photoactive pesticides and
fish poisons (16-20). Representative examples of these compounds
are shown in Figures 1 and 2.

In the search for effective and environmentally nontoxic
biological control agents, the biodegradability of active compounds
is an essential practical consideration. A pesticide or fungicide
which has performed its function should subsequently decompose into
moieties which have no long term effects on the environment. Since
this is a symposium on light-activated pesticides, this discussion
addresses the issues of light stability and photodegradation of
naturally-occurring biocides, particularly photoactive ones, with
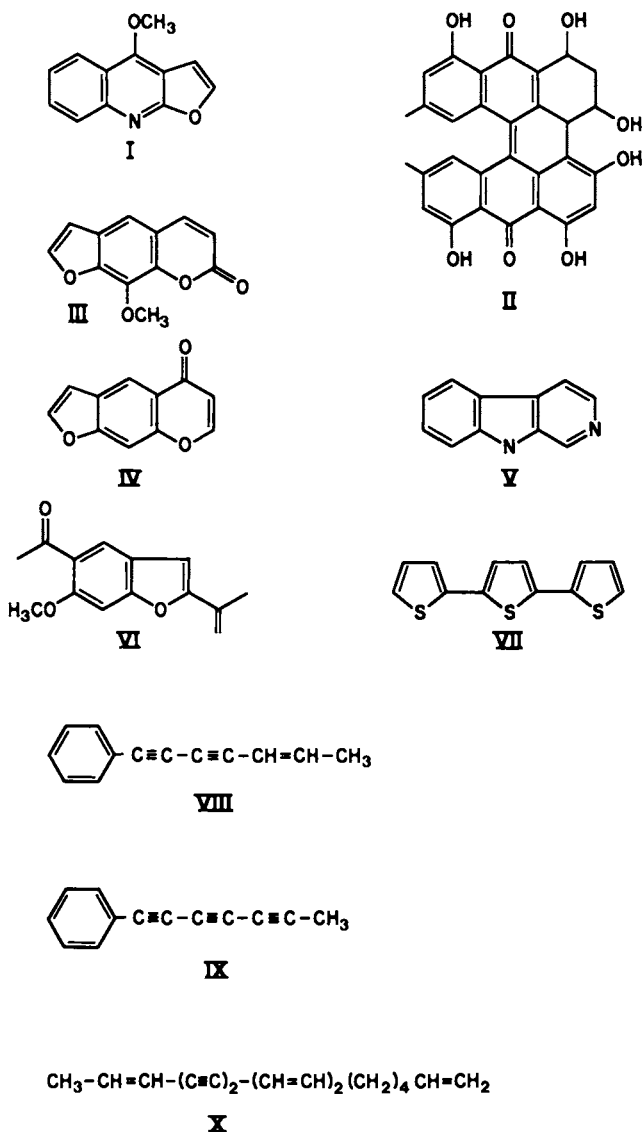
Figure 1. Naturally occurring photoactive compounds. I.
Dictamnine (Dictamnus alba), II. Hypericin (Hypericum
spp.), III. 8-Methoxypsoralen (Rutaceae, Apiaceae), IV.
Khellin (Ammi spp.), V. Harman, VI. 6-Methoxyeuparin
(Encelia spp.), VII. Alpha-terthienyl (Tagetes spp.), VIII.
Phenylheptadiyne ene (Bidens spp.), IX. Phenylheptatriyne
(Bidens spp.), X. Heptadeca tetraene diyne (Bidens spp.).
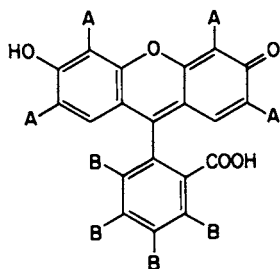
A = I, B = Cl   XI
A = Br, B = Cl   XII
A = I, B = H   XIII
A = Br, B = H   XIV
A = H, B = H   XV

Figure 2. Synthetic xanthene dyes. XI. Rose Bengal, XII.
Phloxin B, XIII. Erythrosin B, XIV. Eosin Yellow, XV.
Fluorescein.

reference to the work on the photodecomposition of the halogenated xanthene dyes.

Degradation of Non-photoactive Natural Pesticides

Many herbicides and pesticides in the environment are degraded by UV radiation from the sun. The exposure of azadirachtin solutions to sunlight caused a rapid decrease in antifeeding potency against first instar larvae of Spodoptera frugiperda (J. E. Smith) and resulted in complete destruction of the compound and its activity after 16 days. HPLC analyses of the exposed solutions showed no traces of azadirachtin (21). The addition of various plant oils, such as neem and castor, to the test solutions afforded some protection (<25%) against photodegradation although whether this was due to the exclusion of oxygen from the medium was not investigated. When applied to soybean leaves in field tests, crude extracts of neem prevented damage to soybean foliage by Popillia beetles for about two weeks. After this period, there was damage comparable to control plants, an observation which was partially attributed to the photodegradation and resultant loss of antifeedant activity of azadirachtin in the crude extracts (22).

In a study of rotenone, the principal insecticidal component of Derris root, Bowman et al. found that fifty percent of the compound in alcoholic solution was photodegraded in light to the demethylated and reduced derivatives rotenolone, dehydrorotenone and rotenonone. Only rotenone at or near the surface of the solutions was available for reaction which suggests that compounds formed at the surface either exclude oxygen and/or diminish light absorption (23). A multitude of other rotenone photodecomposition products with possible carcinogenic properties have also been observed and characterized (24). In addition, some of the components of the pyrethrins found in Chrysanthemum cinerariaefolium (Trevir.) Vis. also degrade in sunlight with subsequent loss of insecticidal activity. Decomposition involves complex isomerization/ rearrangement reactions accompanied by extensive polymerization (25,26).

Photoactive Xanthene Dyes

The light stability of dyed textiles and the properties of dye molecules have been the subject of study for a number of years and numerous reports exist on the photochemical characteristics of dyes which may serve as useful model systems for natural products (27). The photostability of all UV absorbing molecules depends on a number of factors. These include the chemical structure and photophysical properties of the compound, its concentration in the medium, the nature of this medium, light quantity and quality, temperature and other environmental conditions of exposure, and the presence of other reactive species in the medium and/or environment (28). Although the precise role of oxygen in the fading of fabric dyes has been the subject of controversy, under normal exposure conditions the presence of oxygen accelerates the rate of photodecomposition of dyes. It is now generally accepted that the triplet state of many dyes can catalyze the formation of singlet oxygen which leads to the

photooxidation of the substrates in which they are dispersed and even to the self-sensitized photooxidative reactions of azonapthol dyes in low concentrations (27,29,30).

Substituted xanthene dyes, particularly the halogenated fluorescein derivatives, have been shown to be highly photoactive to insects in vitro (16-19). Toxicity is due to the ability of the dyes to absorb light, form triplet excited states and subsequently transfer this energy to form reactive singlet oxygen. Activity increases with the phosphorescence of the dye molecule and with the number and atomic weight of the substituent halogens. These factors increase the relative population of the first excited triplet state of the dye upon illumination and enhance the sensitization of ground state oxygen to the singlet state (31,32) (Figure 2).

Tonogai et al. (20,33) studied the toxicity of four xanthene dyes to fish and found that toxicity was greater after irradiation. The major products of anaerobic photodecomposition were fluorescein and tetrachlorofluorescein which were not as effective as erythrosin, rose bengal, phloxine and eosin in killing fish. Their data show that irradiation in the absence of oxygen causes dehalogenation without the breakdown of the basic xanthene skeleton and suggests that toxicity is most likely caused by the liberated halogens.

Xanthene dyes in aqueous oxygenated solutions photodegrade when exposed to visible light and the rate of degradation depends on oxygen concentration. Photobleaching follows first order kinetics only when dye concentration is low relative to oxygen concentration (31). The visible absorption spectrum of rose bengal disappears completely as photodecomposition occurs and a complex mixture of intermediates and products results. Photodegraded rose bengal does not kill houseflies (Musca domestica) or inhibit the growth of Staphylococcus aureus Rosen. and Bacillus cereus Fr.& Fr. (32). According to Heitz and Wilson (32) who tested the susceptibility of a series of dyes to photodegradation, the two dyes which contain no halogen, rhodamine B and fluorescein (XIV), were most resistant to photodegradation. Iodine and/or bromine atoms on the upper ring system facilitate the reaction while chlorine atoms on the lower ring retard the photodegradation reaction. In addition, susceptibility to photodegradation is positively correlated with phosphorescence of the dye. Molecules which phosphoresce can assume the triplet state more readily and hence catalyze the generation of singlet oxygen.

It is clear that singlet oxygen is responsible for photo-activity as well as for the photodecomposition of the photodynamic xanthene dyes. Nevertheless, many other dyes are susceptible to photobleaching in the absence of oxygen, particularly in the presence of electron donors and in solutions containing species with a readily extractable hydrogen atom. The mechanism of photore-duction often involves the formation of radical or semiquinone intermediates detectable as strong transients and, in the case of azo dyes, may lead to destruction of the chromophore (28,29,34).

Polyacetylenes and Thiophenes

Many polyacetylenes, notably phenylheptatriyne (IX) and its biosynthetic derivative alpha-terthienyl (VII) are also toxic to biological systems in the presence of UV-A radiation (320-400nm) (1,2). Unlike the linear furanocoumarins whose effects can be explained by the photoinduced modification of DNA (8), the lipophilic nature of VII and IX suggests that they may partition into membrane bilayers and thus exert their effects primarily on cell membranes. Alpha-terthienyl acts as a typical Type II photodynamic sensitizer, requiring oxygen for its activity, while photosensitization by phenylheptatriyne occurs under both aerobic and anaerobic conditions (11,35-37). They have been shown to inactivate membrane-bound enzymes and cause increased permeability to K+ ions and subsequent hemolysis in erythrocytes upon irradiation (35,38-40). Both alpha-terthienyl and phenylheptatriyne enhance the permeability of multilamellar liposomes to glucose although evidently by two different mechanisms (41). Specifically, biochemical and biophysical analyses of model membrane systems exposed to UV-A indicate that alpha-terthienyl affects membrane permeability by altering acyl side chains in the hydrocarbon regions in egg-phosphatidylcholine liposomes. Photopolymerization of phenylheptatriyne is the postulated cause of enhanced permeability in distearoylphosphatidylcholine liposomes (42).

In addition to being thermally sensitive, acetylenes are also known to be unstable in light and in aqueous solutions (1,43-45). In a study on the phototoxicity of selected polyacetylenes to the phylloplane yeast Cryptococcus laurentii (Kuff.) Skinner, aqueous solutions of up to 10 µg/mL of test compounds were used to prepare dose response curves in an aerobic microtiter assay (46). Ultraviolet irradiation of longer than five minutes duration seemed to cause breakdown of phenyldiyne-ene (VIII) and the C17 straight chain compound (X), with a concommitant increase in the percent survival of C. laurentii. Alpha-terthienyl (VII) and phenylheptatriyne (IX) were not degraded after 20 minutes of radiation under those test conditions.

In vitro and in vivo degradation experiments by McLachlan et al. (36) showed that straight chain acetylenes decomposed most rapidly followed by aromatic acetylenes, and lastly, thiophenes. Photodecomposition was marked by a collapse of the UV spectra of VII and IX and by the rapid formation and disappearance of intermediate species in other compounds. Although the photodegradation of straight chain and aromatic acetylenes does not require oxygen, thiophene decomposition is aerobic. The authors suggest that there is greater efficiency in the transfer of impinging light energy to singlet oxygen in thiophenes and that a nonphotodynamic process competes successfully with singlet oxygen generation in the other polyacetylenes resulting in a higher number of molecular rearrangement events.

Conclusions

Numerous details are known about naturally-occurring photosensitizers and their potent in vitro effects on biological

systems. These properties have been exploited in some cases, for example, 8-methoxypsoralen (III) has been used to treat pscriasis, vitiligo and other skin disorders for a number of years (47), and recently, a patent application for the possible commercial use of alpha-terthienyl as a mosquito and blackfly larvicide was filed (48). Polyacetylenes and thiophenes are toxic to numerous organisms at low concentrations and their potential as commercial insect-icides, fungicides, piscicides and molluscides is further enhanced by evidence of rapid biodegradability of most compounds in aqueous solution and sunlight. The chemical nature and lifetimes of photo-decomposition intermediates and products has not been thoroughly explored but clearly this must be addressed before these compounds can be used in the field.

Literature Cited

1. Towers, G. H. N. Can J. Bot. 1984, 62, 2900-11.
2. Towers, G. H. N. J. Chem. Ecol. 1986, 12, 813-21.
3. Downum, K. R.; Rodriguez, E. J. Chem. Ecol. 1986, 12, 823-34.
4   Knox, J. P.; Dodge, A. D. Phytochemistry, 1985, 24, 889-96.
5. Towers, G. H. N.; Abramovski, Z. J. Nat. Prod. 1983, 46, 576-81.
6. Ashwood-Smith, M. J.; Towers, G. H. N.; Abramovski, Z. ; Poulton, G. A.; Liu, M. Mutat. Res. 1982, 102, 401-12.
7. Proksch, P.; Proksch, M.; Towers, G. H. N.; Rodriguez, E. J. Nat. Prod. 1983, 46, 331-34.
8. Song, P.-S.; Tapley, K.J. Jr. Photochem. Photobiol. 1979, 29, 1177-97
9. Berenbaum, M. Science 1978, 201, 532-34.
10. Abeysekera, B. F.; Abramovski, Z.; Towers, G. H. N. Photochem. Photobiol. 1983, 38, 311-15.
11. Arnason, J. T.; Wat, C. K.; Downum, K. R.; Yamamoto, E.; Graham, E.; Towers, G. H. N. Can. J. Microbiol. 1980, 26, 698-705.
12. Champagne, D. E.; Arnason, J. T., Philogene, B. J. R.; Morand, P.; Lam, J. J. Chem. Ecol. 1986, 12, 835-58.
13. Hudson J. B.; Graham, E. A.; Towers, G. H. N. Photochem. Photobiol. 1982, 36, 181-86.
14. Marchant, Y. Y.; Towers, G. H. N. Biochem. Syst. Ecol. 1986a, in press.
15. Downum, K. R. In Natural Plant Resistance to Pests: Roles of Allelochemicals; Green, M.; Hedin, P. A., Eds.; American Chemical Society, Washington, DC, 1986; pp 197-205.
16. Carpenter, T. L.; Respicio, N. C.; Heitz, J. R. Environ. Entomol. 1984, 13, 1366-70.
17. Carpenter, T. L.; Mundie, T. G.; Ross, J. H.; Heitz, J. R. Environ. Entomol. 1981, 10, 953-56.
18. Pimprikar, G. D.; Fondren, J. E. Jr.; Heitz, J. R. Environ. Entomol. 1980, 9, 53-8.
19. Heitz, J. R. In Insecticide Mode of Action; Coats, J. R., Ed.; Academic Press, New York, 1982; pp 429-57.
20. Tonogai, Y.; Ito, Y.; Iwaida, M.; Tati, M.; Ose, Y.; Sato, T. J. Toxicol. Sci. 1979, 4, 115-26.

21. Stokes, J. B.; Redfern, R. E.   J. Environ. Sci. Health 1982, A17, 57-65.
22. Ladd, T. L. Jr.; Jacobson, M.; Buriff, C. R.   J. Econ. Entomol. 1978, 71, 810-14.
23. Bowman, M. C.; Holder, C. L.; Bone, L.I.   J. Assoc. Off. Anal. Chem. 1978, 61, 1445-55.
24. Cheng, H. M.; Yamamoto, I.; Casida, J. E.   J. Agric. Food Chem. 1972, 20, 850-6.
25. Bullivant, M. J.; Pattenden, G. Pyrethrum Post 1973, 13, 64-75.
26. Bullivant, M. J.; Pattenden, G. Pyrethrum Post 1971, 11, 72-9.
27. Sinclair, R. Y.   Photochem. Photobiol. 1980, 31, 627-9.
28. Evans, N. A.; Stapleton, I. W. In Chemistry of Synthetic Dyes, Venkataraman, K. Ed.; Academic Press, New York, 1978, 8, 221-77.
29. McKellar, J. F.   Radiat. Res. Rev. 1971, 3, 141-65.
30. Bentley, P.; McKellar, J. F.   Rev. Prog. Coloration 1974, 5,33-48.
31. Wilson, W. W.; Heitz, J. R.   J. Agric.Food Chem. 1984, 32, 615-17.
32. Heitz, J. R.; Wilson, W. W. In Disposal and Decontamination of Pesticides, Kennedy, M. V., Ed.; American Chemical Society, Washington, DC, 1978, pp 35-48.
33. Tonogai, Y.; Iwaida, M.; Tati, M.; Ose, Y.; Sato, T.   J. Toxicol. Sci. 1978, 3, 205-14.
34. Kellman, A.;. Lion, Y.; Photochem. Photobiol. 1979. 29, 217-22.
35. Wat, C. K.; MacRae, W. D.; Yamamoto, E.; Towers, G. H. N.; Lam. J. Photochem. Photobiol. 1980, 32, 167-72.
36. McLachlan, D.; Arnason, J. T.; Lam, J. Photochem. Photobiol. 1984, 39, 117-82.
37. Weir, D.; Scaiano, J. C. ; Arnason, J. T.; Evans, C. Photochem. Photobiol. 1985, 42, 223-30.
38. Yamamoto, E.; Wat, C. K.; MacRae, W. D.; Towers, G. H. N.   FEBS Lett. 1979, 107, 134-6.
39. Bakker, J.; Gommers, F. J.; Nieuwenhuis, I.; Wynberg, H.   J. Biol. Chem. 1979, 254, 1841-4.
40. MacRae, W. D.; Irwin, D. A. J. Bisalputra, T.; Towers, G. H. N. Photobiochem. Photobiophys. 1980, 1, 309-18.
41. McRae, D. G.; Yamamoto, E.; Towers, G. H. N.   Biochim. Biophys. Acta 1985, 821, 488-96.
42. McRae, D. G.; Webb, M.; Marchant, Y. Y.; Towers, G. H. N.; Thompson, J. E.   Biochim. Biophys. Acta 1986, submitted.
43. Anchel, M.; Polatnick, J.; Kavanagh, F.   Arch. Biochem. 1950, 25, 208-20.
44. Celmer, W. D.; Solomons, I. A.   Amer. Chem. Soc. J. 1953, 751, 372-76.
45. Bohlmann, F.; Burkhardt, T.; Zdero, C.   Naturally Occurring Acetylenes, Academic Press, London, 1973.
46. Marchant, Y. Y.; Towers, G. H. N.   Biochem. Syst. Ecol. 1986b. in press
47. Warin, A. P., Carruthers, J. A.   Clin. Exptl. Dermatol. 1976, 1, 181-189.
48. Towers,. G. H. N.; Arnason, J. T.; Wat, C. K.; Lambert, J. D. H. Can. Pat. 1984, 1, 173,743.

Chapter 12

α-Terthienyl as a Photoactive Insecticide: Toxic Effects on Nontarget Organisms

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Alpha-terthienyl is a highly potent photoinsecticide. Laboratory experiments demonstrated that it produces light-dependent toxic effects in non-target organisms. The results obtained with the fish Pimephales promelas (fathead minnow), tadpoles of Rana pipiens and Hyla crucifer, and water fleas (Daphnia magna) are reviewed. They cast a doubt upon the published claim that alpha-terthienyl had an activity against non-target organisms low enough to allow its use for mosquito control in the field. Greater selectivity was encountered with other phototoxic molecules (Figure 1).

Although the ideal pesticide is expected to display perfect selectivity against its intended target organism, few commercially available pesticides do, unfortunately. Toxicity against non-target organisms, including humans, must therefore be determined under realistic conditions in order to decide whether the potential benefits in the use of a new product outweigh the risks.

Light-dependent insecticides have had limited commercial use to date, and little is known about their selectivity. Alpha-terthienyl, 1, is a molecule which has displayed toxicity in a variety of organisms, such as bacteria, viruses, fungi, nematodes, human erythrocytes and human skin, eggs and larvae of insects, algae and plants (1). However, it was reported at a recent symposium that, in field applications, alpha-terthienyl used in mosquito control had very low activity against non-target organisms (2). Because we had already described the light-dependent toxicity of the compound in Rana pipiens tadpoles (3), and because for a number of years we had routinely used aquatic organisms for monitoring the activity of new phototoxic molecules, we thought that a critical survey of the phototoxicity of alpha-terthienyl in non-target organisms was desirable. In this review we comment on the light-dependent toxicity of 1 in the mosquito Aedes aegypti, and compare it to that in other environmentally relevant aquatic organisms. The laboratory
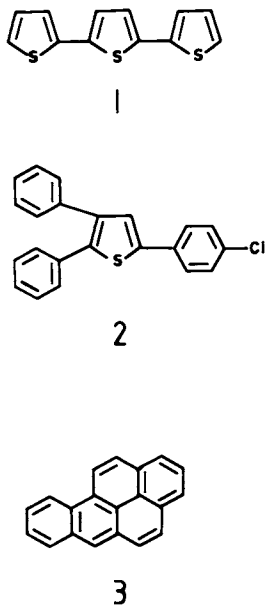
Figure 1. Structure of the phototoxic compounds mentioned.

data were obtained by placing the organisms into water containing
the desired amount of sensitizer, waiting up to 1 h, and placing the
vessel under a bank of 8 tubes (RPR-3500A from the Southern New
England Ultraviolet Co, Hamden, CT) emitting at 320-400 nm, with a
maximum at 350 nm. The tubes were placed horizontally 7 to 9 cm
above the water, and the light intensity at this distance was about
13 W m^{-2}. The irradiation time ranged from 30 min to 1 h. While our
experiments were not intended to replace actual exposure of the
organisms to sunlight under environmentally relevant conditions,
they were meant to provide a ranking of the sensitivity of the
different organisms exposed to UV light under similar, reproducible,
conditions. The results should be qualitatively similar to those
obtained with sunlight.

The Photoinsecticidal Activity of Alpha-Terthienyl

We used the mosquito Aedes aegypti for testing the light-dependent
activity of alpha-terthienyl under conditions somewhat different
from those reported in earlier studies (4, 5). In order to deter-
mine acute toxicity data and to follow the development of organisms
which had been treated at specific stages, we used a procedure
recently tested with other photosensitizers (6). Egg sheets were
placed in water in the morning and kept in an incubator at 28 °C. In
the afternoon first instar larvae were collected, and were incubated
overnight at room temperature in the presence of 1. After 30 min of
irradiation with UV light (320-400 nm), the surviving larvae were
placed in petri dishes with additional water, fed, and observed
daily until adults emerged in dark controls. From the shape of the
survival curves at different initial concentrations, we intended to
determine the extent and timing of any delayed toxicity. However,
practically all the larvae which survived 24 h reached adulthood
(7). Figure 2 shows the survival profiles at 0.005 mg/L, the
highest concentration at which survival was observed, and at 0.0009
mg/L compared to 0.03 mg/L in the dark (very similar to the profile
obtained with untreated larvae). Except for the amplitudes, the
curves are very similar in shape. The absence of delayed effects
suggest that the mechanism of phototoxicity does not involve a
drastic modification of nucleic acids, which would have been likely
to impair molting and/or emergence to adults. The conclusion that
the light-dependent toxicity of alpha-terthienyl results from damage
to membranes rather than to nucleic acids is supported experimen-
tally by the recent results of Tuveson et al. with bacteria (8). The
absence of delayed toxicity which we observed with alpha-terthienyl
also contrasts with the delayed effects registered with furano-
coumarins in similar experiments (6). These compounds are known to
produce photoadducts with DNA.
     Fourth instar larvae, not surprisingly, were much more
resistant to the photosensitized treatment than first instar larvae.
Our LC$_{50}$ values were 0.002 and 0.45 mg/L with 1st and 4th instar
larvae respectively, in general accord with the results of Arnason
et al (4) and Wat et al. (5). A comparison of the phototoxic levels
of 1 in A. aegypti and A. intrudens under identical conditions has
not been made with first-instar larvae, but there seems to be quali-
tative agreement for older larvae of these two species (9).
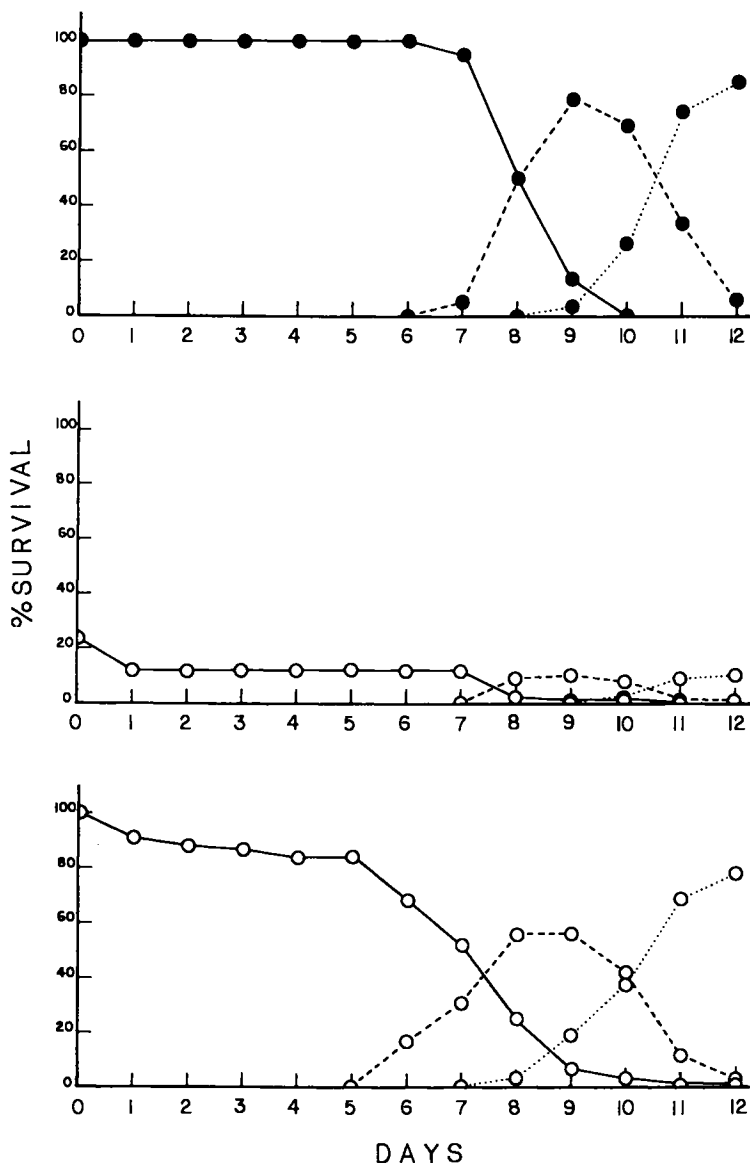
Figure 2.   Surviving larvae (solid lines), pupae (broken lines)
and adults (dotted lines) obtained from first-instar larvae or
A. aegypti in the dark (closed circles) and irradiated for 30
min. (open circles), as a function of time. The larvae were
incubated overnight with 1 prior to their irradiation. The
concentrations of 1, which were 0.03 (top), 0.005 (middle), and
0.0009 mg/L (bottom), are shown on a logarithmic scale. (Repro-
duced with permission from Reference 7. Copyright 1987 Plenum.)

Since older larvae were more resistant to the light-dependent effect of alpha-terthienyl than younger ones, we expected that treating still younger larvae would produce more dramatic results. This expectation was not fulfilled. For example, larvae about 2-h old were treated as above with alpha-terthienyl, except that the incubation time was shortened from about 14 h to 30 min, while the irradiation time was kept at 30 min. In these experiments the larvae were much younger when irradiated, but their exposure to the sensitizer was correspondingly shorter. The LC_{50} values observed 24 h later were 0.075 mg/L in the dark controls, and 0.0016 in the exposed larvae.

Measurement the phototoxicity of 1 toward mosquito eggs was more delicate because this compound has an appreciable toxicity to larvae in the dark, an effect likely to be at its maximum with newly hatched larvae. By performing microscopic examinations of the eggs, we established that 1 was not phototoxic at concentrations up to 6.7 mg/L. At this concentration, however, all the newly formed larvae were killed by the sensitizer, but in a light-independent manner. We note that the lack of photoovicidal activity in A. aegypti is not a general characteristic of 1, since a very high photoovicidal activity has been documented in Drosophila melanogaster (10).

Unexpected results came from the study of the pupae, believed to be immune to photodamage by 1 (4). Our initial results indeed agreed with this conclusion since all the pupae were still alive 24 h after phototreatment with 6.7 mg/L of 1, but highly irreproducible results were obtained when the irradiated pupae were observed through adult emergence. Finally, we recognized that the age of the pupae at the time of treatment was an important variable, and determined the survival curves shown in Figure 3. At 1 or 2 days of age, the pupae were quite sensitive to the phototreatment with 1 (LC_{50} = 0.06 mg/L), but three-day-old pupae were no longer affected. To our knowledge, this photoinduced effect of 1 is the first example of insect pupae killed with a photoactive insecticide. Since pupae are usually quite resistant to pesticides, this activity makes 1 an even more interesting photopesticide than originally suspected.

The Photoactivity of Alpha-Terthienyl in Tadpoles

Several years ago, we observed that 1 was phototoxic in the immature frog Rana pipiens (3). The experiments were performed with up to 2 h of sunlight, which is highly variable in our area, and the survival was recorded immediately after irradiation. Even with such limited exposure, 1 showed significant toxicity. The LC_{50} value for acute phototoxicity was 0.065 mg/L after 30 min, and 0.025 mg/L after 2 h of irradiation.

Wishing to obtain more complete data, we recently collected tadpoles comparable in development to those of R. pipiens used earlier. They turned out to be tadpoles of Hyla crucifer, and they were used for measuring the phototoxicity of 1 in sunlight and in UV light. In these experiments, the initial incubation time was 1 h and the irradiation time was also 1 h, but the survival was determined 24 h after treatment. Again, although these are short durations compared to those likely to be faced by organisms in nature, the LC_{50} values were very impressive, about 0.003 mg/L in both series of

experiments (Figure 4). This phototoxicity level of 1 is comparable to the 24-h survival value (0.002 mg/L) observed with the first-instar larvae of A. aegypti (7).

   The analysis of the phototoxicity of 1 in immature mosquitos from different genera and/or different species under carefully controlled conditions has not yet been reported. It was found (Borovsky, Linley, and Kagan, unpublished results) that, in the presence of sunlight, the difference in phototoxic response in larvae of A. aegypti, A. taeniorhynchus, and Culex quinquefasciatus was quite large, with A. aegypti being the most affected at the lowest concentrations. A recent report gave a 24-h LC_{50} value of 0.0275 mg/L when A. atropalpus was exposed to 1 and UV light (11). Because of differences in conditions (light intensity, duration of the exposures, age of the larvae), direct comparison with our work is meaningless.

Phototoxicity of Alpha-Terthienyl in Fish

We investigated the toxicity of 1 in fish using the fathead minnow (Pimephales promelas) (12), which is commonly available and has been used extensively in toxicology. Again, our experimental conditions did not approximate field conditions, which would have provided much longer exposures. No toxicity was observed at concentrations as high as 10 mg/L when the fish were kept in the dark. However, with 30 min of incubation followed by 30 min of irradiation, the 24-h LC_{50} value was found to be about 0.05 mg/L when UV light was used, and about 0.02 mg/L when sunlight was used. In order to calibrate the magnitude of this effect, we measured on our fish the light-independent toxicity of rotenone, one of the best-known fish poisons, and found its 24-h LC_{50} value to be 0.04 mg/L. In other words, 1 is a fish poison at least twice as potent as rotenone.

   We were interested in determining how the exposure time to 1 before irradiation affected the survival of the fish. Longer incubations may be expected to increase the amount of sensitizer picked up by the fish (thereby increasing the toxicity), but they also allow more time for depuration (thereby decreasing the toxicity of the sensitizer). The actual survival profile showed a minimum at 2 h (Figure 5), as would be expected if the depuration process were slower than the uptake, taking into account the limited amount of sensitizer available to the fish (1). The term depuration has been used here to imply the clearing of the toxin from the organisms' system, without regard for the actual mechanisms, which could involve, for example, detoxification and/or excretion. Further work on the mechanism of this depuration process will be highly desirable.

   The mechanism for the phototoxicity of 1 in fish is not known. However, we established that direct contact was important. In these experiments (1), water fleas (Daphnia magna) were exposed to known doses of 1 prior to being fed to fish which, in turn, were exposed to UV light. Negligible phototoxicity was observed in the treated fish.
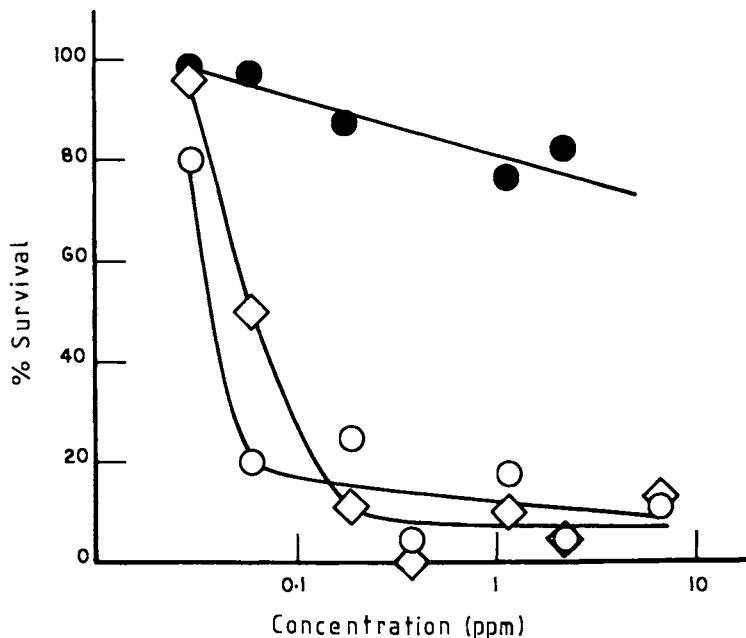
Figure 3. Survival of A. aegypti pupae through adult emergence,
following a 30-min. incubation and 30-min. irradiation in the
presence of 1. The age of the pupae at the time of irradiation
was 0-1 (open circles), 1-2 (open squares), and 2-3 day (closed
circles). (Reproduced with permission from Reference 7. Copy-
right 1987 Plenum.)
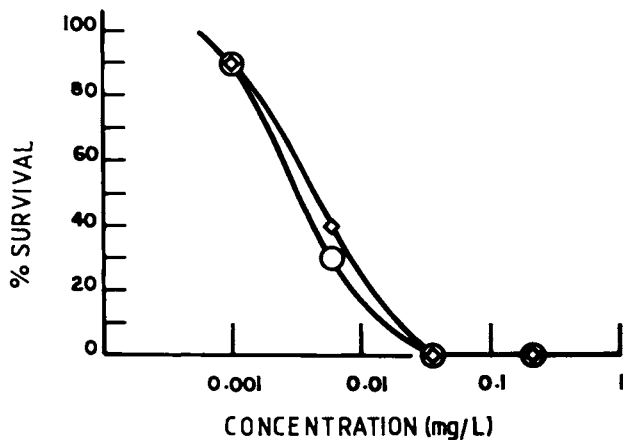
Figure 4. Survival (24 h) of immature Hyla crucifer as a function
of the concentration of 1 (on a logarithmic scale). The
incubation time was 1 h, and the irradiation 1 h, with sunlight
(circles) or with UV (squares).

Phototoxicity of 1 in Daphnia magna

The activity of 1 was also measured in the water flea D. magna, a standard organism in toxicological studies. In the dark, no toxicity was noticed at concentrations below 7 mg/L and, in the control experiments, the UV light alone had no effect on the survival of the organisms. In the light-dependent studies, the organisms were incubated for 1 h with 1, and then irradiated with UV light for 1 h. The survival was recorded 24 h later. The LC_{50} value in these conditions was 0.0013 mg/L (Figure 6) (12).

Mechanistic considerations

The detailed biological mechanism for the phototoxicity of 1 in aquatic organisms is completely unknown. In another example of phototoxicity in fish, that of anthracene, extensive damage to the skin and gills were reported (13). Although the two sensitizers appeared to generate the same type of response in our fish, a detailed analysis remains to be conducted. On a molecular level, it is reasonable to expect that 1, an excellent singlet oxygen sensitizer (14), damages cell components through oxidative processes. The results obtained with A. aegypti suggest that nucleic acids are not affected, since very little delayed mortality was observed. In view of other examples where cell membranes were damaged by 1, such as in human erythrocytes (15), lipid damage may be expected. Recent results from Tuveson et al. provide additional confirmation, since the same kinetics of photoinactivation with 1 were found in four mutant strains of E. coli which contained all four possible combinations of genes controlling excision proficiency and sensitivity to oxidative damage (8).

   We also attempted to protect organisms from the phototoxic effects of 1 by using aqueous solutions containing beta-carotene, a standard singlet oxygen quencher known to react at diffusion-controlled rates (16).   Little or no protection could be demonstrated in either mosquito larvae or Daphnia, unless a large excess of carotene was used. For example, fourth instar larvae of A. aegypti were incubated for 30 min in a 0.19 mg/L solution of 1 in the presence of varying amounts of beta-carotene. Figure 7 shows the 24-h survival curve, indicating that about 30 mg/L of beta-carotene produces 50% protection, about an 80-fold molar excess of beta-carotene over 1. Similar studies with Daphnia in 5 ug/L of 1 showed that 50% protection was produced by 50 mg/L of beta-carotene, nearly a 5000-fold molar excess (Figure 8). In these two examples, it is likely that standard singlet oxygen quenching was not a prominent reaction; perhaps the protecting effect of beta-carotene was simply due to filtering of the UV light, an application of Beer's law. It is useful to note in this regard that singlet oxygen quenching experiments have usually been much less successful when performed in vivo than in vitro. In an extreme case, some singlet oxygen quenchers added to the diet were observed to enhance even the phototoxic effect of xanthene dyes in flies, although a very modest protection with beta-carotene was noted (17).
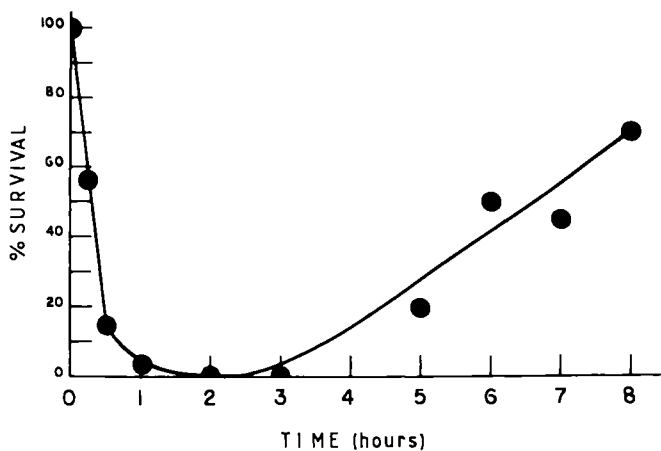
Figure 5. Percent survival of <u>P. promelas</u> as a function of incubation time. In all cases the concentration of 1 was 0.1 mg/L, and the irradiation time 30 min. The survival was recorded 24 h after the irradiations. (Reproduced with permission from Ref 12. Copyright 1987 Pergamon)
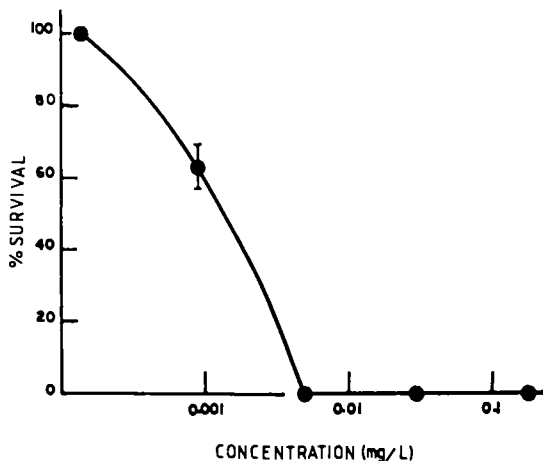
Figure 6. Survival (24 h) of <u>D. magna</u>, with 1 h incubation and 1 h irradiation. The concentration scale for 1 is logarithmic. (Reproduced with permission from Ref 12. Copyright 1987 Pergamon)
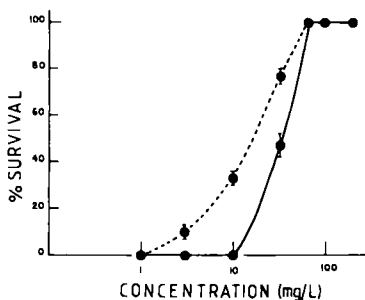
Figure 7. Survival of fourth-instar larvae of A. aegypti incubated with 1 (0.02 mg/L) for 30 min and irradiated for 30 min in the presence of varying concentrations of beta-carotene (shown on a logarithmic scale), 1 h (broken line) and 24 h (solid line) after irradiation. (Reproduced with permission from Ref 12. Copyright 1987 Pergamon)
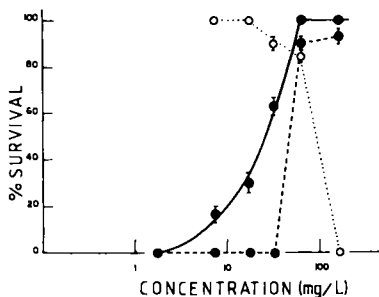
Figure 8. Survival of D. magna incubated for 1 h in the presence of 1.2 mg/L of 1, and irradiated for 1 h. The experiments were conducted in the presence of varying concentrations of beta-carotene (shown on a logarithmic scale), and the results were recorded immediately after irradiation (solid line) and 24 h later (broken line). The dotted line represents the survival in the dark, in the absence of 1. (Reproduced with permission from Ref 12. Copyright 1987 Pergamon)

Alpha-terthienyl As A Phototoxic Insecticide: Is It Selective?

The results of our laboratory experiments suggest that 1 is not a selective photoinsecticide, that it has very significant activity in aquatic organisms, and that it is premature to advocate its use for mosquito control.

Opposite views have been expressed (2, 18). Two points at issue in that work concern the interpretation of the very high photo-toxicity in Daphnia (higher than in mosquito larvae) and the lack of phototoxicity of 1 in trout, in relation to the suitability of the chemical for field use. The statement (18) that "under laboratory conditions Daphnia .... survived 1 and UV treatment without any significant or visible signs of intoxication" is supported neither by the original results (18) nor by ours.

Further studies may be required before passing a final judgment on the suitability of 1 for insect control. It is conceivable that trout have some protection or depuration mechanism not available to fathead minnows. However, we believe that the discrepancy is more likely to be associated with the difference in protocols used for exposing the fish to the chemical. Instead of adding an ethanol solution to the water containing the fish (18), we used dimethyl-sulfoxide as carrier for the very hydrophobic 1, and we mixed the water thoroughly before adding the fish. The compound precipitates out of solution almost immediately in the former procedure, but apparently not in the latter. In the absence of mechanical mixing, the sensitizer which precipitates forms a film on the water, and any fish remaining away from the surface can be immune from phototoxic effects during short-term experiments. In small vessels under laboratory conditions, we could not demonstrate any significant difference in the outcome whether EtOH or DMSO was used. Even with a modest scale-up of the holding vessel (from 400 to 1000 mL), the fish frequently swam by the surface, and all displayed equal susceptibility to the light treatment. In larger tanks or under field conditions, however, particularly in the absence of wind or currents, the results perhaps could be quite different.

Finally, the kinetics of the various processes at play cannot be ignored. To a fish remaining at a fixed position in the water, the risks of exposure are time-dependent, during the period when the chemical precipitating at the surface establishes equilibrium with the bulk of the water (in such experiments, the ratio of surface area to volume of the solution should be quite important). Irradiated before the sensitizer had a chance of reaching it, a fish will survive. Introduced directly into an homogeneous solution of the sensitizer, this fish will be at much greater risk much sooner. The difference will also depend on the time span between the introduction of the chemical and the beginning of the experiment, which includes the actual contact time of the fish with the chemical.

In our experiments we placed 5 fish in an homogeneous mixture of 1 in 0.4 L of water and irradiated 0.5 h later. In the work with trout 1 was added to the surface of ca. 40 L of water containing 10 fish which were irradiated 1 h later (18). The lack of phototoxicity for 1 reported in trout could be simply the result of insufficient contact between the chemical and the organisms, rather than to biological immunity.

It is interesting to note that while the precipitation of 1 which occurs when an ethanol solution is added to water should decrease the phototoxic effects in fish and most other aquatic organisms, it should actually enhance the phototoxicity in mosquito larvae. Since these must come to the surface of the water in order to breathe, they are exposed there to disproportionately high concentrations of the hydrophobic chemical.

Under natural conditions, consideration must be given to situations not easily controlled in laboratory or field trials. For example, phototoxic damage to organisms obviously depends upon their exposure to UV light. In waters whose absorption coefficient follows a steep vertical gradient, the organisms tested would gain protection by remaining near the bottom during daytime. They could also gain some protection by remaining in shaded areas or under shelters. Finally, some components of natural waters could perhaps provide protection from photodynamic damage by acting as quenchers, affecting either the lifetime of a sensitizer's excited states or that of singlet oxygen or other reactive products which might be the actual toxic agents.

The rate of depuration is another factor important to the survival of an aquatic organism in contact with a photosensitizer. Since little is known in this area, we placed fathead minnows in a solution containing 0.05 mg/L of 1. After 30 min, they were divided into two groups. One group was irradiated for 30 min, and 50% of these fish were dead 24 h later. The other group was transferred into clean water and kept there for various lengths of time before exposure to UV light. As shown in Figure 9, the photosensitizing effect of 1 wore off rapidly: the fish were no longer at risk after 3 h of depuration. This observation should give optimism to fish which become accidentally exposed to a photoactive pesticide. By heading toward unpolluted water, particularly under cover of darkness, they stand a good chance of full recovery. Likewise, other aquatic organisms may well have rapid depuration mechanisms. However, all may not be equally able to escape rapidly from a contaminated area and to avoid exposure to light before completing the depuration process. For example mosquito larvae, which must frequently come to the surface in order to breathe, are very unlikely to travel as fast as fish away from contaminated surface water toward deeper and cleaner areas. Here again, the dynamic aspects of the photosensitization under non-equilibrium conditions are highly important, and they provide some target selectivity in the use of light-activated pesticides as mosquito larvicides.

Conclusions

To our knowledge, there is not yet one molecule whose phototoxicity has been proved to be restricted to one single target organism. The statement that "1 is a highly effective larvicide with acceptable nontarget effects" (18) needs further objective evaluation. It would be particularly important to obtain a full set of data for target as well as for non-target organisms under field conditions.

In our recent studies, we encountered two other phototoxic molecules which, under identical experimental conditions, produced
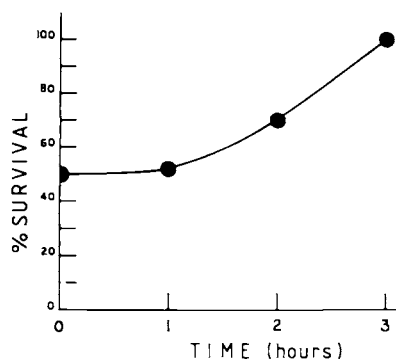
Figure 9. Survival (24 h) of <u>P. promelas</u> exposed to 1 (0.1 mg/L) for 30 min, transferred into clean water, and irradiated for 30 min after the delay shown.

exactly the same LC_{50} value as 1 in first-instar larvae of A. aegypti. They are 5-(4-chlorophenyl)-2,3-diphenylthiophene (2) and benzo[a]pyrene (3). The former (UBI-T930, Micromite) has an extremely low toxicity in mammalian organisms, and was developed by Uniroyal Co. as an acaricide (19-20). The latter, on the other hand, is a well-known carcinogen. In some recent work we demonstrated that, contrary to earlier opinions, phototoxicity in polycyclic aromatic hydrocarbons (PAH's) is not necessarily associated with their carcinogenicity, and that the environmental impact of pollution with PAH's associated with their light-dependent activity remains to be assessed (21-23).

Comparing the phototoxicity of 1, 2, and 3, we have uncovered a degree of selectivity which suggests that further research designed to amplify this property could be profitable. It is particularly striking with respect to the fish P. promelas. While 1, in the presence of UV, ranks among the very best fish poisons known, we could not demonstrate any phototoxicity with 2 and 3 under the same conditions (this represents a difference in activity of at least three orders of magnitude). Figure 10 also illustrates another example of differential phototoxicity, observed with tadpoles of H. crucifer. There is a difference of roughly one order of magnitude in the LC_{50} values for 24-h survival in going from 1 (LC_{50} = 0.02 mg/L), to 3 (LC_{50} = 0.4 mg/L), and to 2 (LC_{50} = 3 mg/L).

Future research on the design of photoactive insecticides will certainly lead to more active compounds possessing greater selectivity. In such studies, the discovery of molecules without activity in one important organism, despite favorable spectral properties, may turn out to be of greater value than the finding of yet one more phototoxic compound.
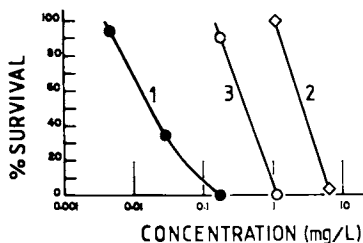
Figure 10. Survival (24 h) of tadpoles of H. crucifer exposed to 1 (solid circles), 2 (open circles), and 3 (squares) for 1 h and irradiated for 1 h. The concentration scale is logarithmic.

Literature Cited

1. Kagan, J.; Kagan, E. D.; Seigneurie, E. Chemosphere 1986, 15, 49–57, and reference cited.
2. Philogene, B. J. R.; Arnason, J. T. National Meeting of the Entomological Society of America, Symposium on light-dependent interactions between plants and insects, San Antonio, Texas, Dec 12, 1984.
3. Kagan, J.; Kagan, P. A.; Buhse, Jr., H. E. J. Chem. Ecol. 1984, 1115–1122.
4. Arnason, J. T.; Swain, T.; Wat, C. K.; Graham, E. A.; Partington, S.; Lam, J.; Towers, G. H. N. Biochem. Syst. Ecol. 1981, 9, 63–68.
5. Wat, C. K.; Prasad, S.; Graham, E.; Partington, S.; Arnason, T.; Towers, G. H. N.; Lam, J. Biochem. Syst. Ecol. 1981, 9, 59–62.
6. Kagan, J.; Szczepanski, P.; Bindokas, V.; Wulff, W. D.; McCallum, J. S. J. Chem. Ecol. 1986, 12, 899–914.
7. Kagan, J.; Kagan, E. D.; Patel, S.; Perrine, D.; Bindokas, V. J. Chem. Ecol. 1987, 13, 593–604.
8. Tuveson, R. W.; Berenbaum, M. R.; Heininger, E. E. J. Chem. Ecol. 1986, 12, 933–948.
9. Philogene, B. J. R.; Arnason, J. T.; Berg, C. W.; Duval, F.; Champagne, D.; Taylor, R. G.; Leitch, L. C.; Morand, P. J. Econom. Entomol. 1985, 78, 121–126.
10. Kagan, J.; Chan, G. Experientia 1983, 39, 402–403.
11. Arnason, J. T.; Philogene, B. J. R.; Berg, C.; MacEachern, A.; Kaminski, J.; Leitch, L. C.; Morand, P.; Lam, J. Phytochem., 1986, 1609–1611.
12. Bennett, W. E.; Maas, J. L.; Sweeney, S. A.; Kagan, J. Chemosphere 1986, 15, 781–786.
13. Bowling, J. W.; Leversee, G. J.; Landrum, P. F.; Giesy, J. P. Aquat. Toxicol. 1983, 3, 79–90.
14. Reyftmann, J. P.; Kagan, J.; Santus, R.; Morliere, P. Photochem. Photobiol. 1985, 41, 1–7.
15. MacRae, W. D.; Irwin, D. A. J.; Bisalputra, T.; Towers, G. H. N. Photobiochem. Photobiophys. 1980, 1, 309–318.
16. Foote, C. S. In Singlet Oxygen; Wasserman, H. H. and Murray, R. W., Eds.; Academic: New York, 1979; p 139.
17. Robinson, J. R.; Beatson, E. P. Pest. Biochem. Phys. 1985, 24, 375–383.
18. Philogene, B. J. R.; Arnason, J. T.; Berg, C. W.; Duval, F.; Morand, P. J. Chem. Ecol. 1986, 12, 893–898.

19.   Relyea, D. I.; Hubbard, W. L.; Grahame, Jr., R. E. U.S. Patent
      4,174,405, 1979.
20.   Relyea, D. I.; Moore, R. C.; Hubbard, W. L.; King, P. A. Proc.
      10th Int. Congr. Plant Prot., Vol. 1, Croydon, U.K., p 355.
21.   Kagan, J.; Kagan, E. D., Kagan, P. A. Chemosphere 1985, 14,
      1829-1834.
22.   Kagan, J.; Kagan, E. D., Kagan, P. A., In Aquatic
      Photochemistry; Cooper, W. J.; Zika, R. G., Eds.; ACS Symposium
      Series; American Chemical Society: Washington, DC, 1986; in
      press.
23.   Kagan, J.; Kagan, E. D. Chemosphere 1986, 15, 243-251.

Chapter 13

Using Bacterial Mutants and Transforming DNA To Define Phototoxic Mechanisms

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Mutant Escherichia coli K12 strains can be used to
determine whether phototoxins damage cells by attacking
DNA or cell membranes and to assess the ability of
phototoxins to induce excision repair and error-prone
repair which leads to mutation. The role of fatty
acids versus essential membrane proteins as potential
lethal targets for those phototoxins attacking the
membrane can be evaluated using an E. coli fatty acid
auxotroph. Inactivation of Haemophilus influenzae
transforming DNA can evaluate DNA as a lethal target
for a phototoxin.

Microorganisms have played a central role in the development of
current ideas concerning the mechanisms that underlie transmission
of hereditary information, gene structure, and the regulation of
gene function. Analyses involving Escherichia coli and its viruses
have been particularly important in these developments. E. coli is
probably the best understood system in the biological world (1)
partly because: 1) under optimal conditions, cell division can
occur every twenty (20) minutes, which provides for completion of
experiments in hours rather than days; 2) the minimal medium
required for growth of wild type E. coli is simple, consisting of
salts plus a carbon source (e.g., glucose); 3) E. coli is a haploid
organism; therefore, mutations are expressed directly without the
need for complex matings to produce strains homozygous for induced
mutations; 4) E. coli can be mated, and in combination with general-
ized transduction involving bacteriophage P1, sophisticated genetic
analyses can be performed. Genetic manipulations are easy, and
strains may be constructed for specific purposes (e.g., the study of
the biological effects of phototoxins); 5) the techniques for
cloning both eucaryotic and procaryotic genes involve E. coli and
its mutants.

E. coli -- DNA Repair Systems

E. coli is particularly useful for the study of phototoxins because its mechanisms for the repair of radiation- and chemical-induced damage have been characterized in detail (2, 3). A minimum of six or seven E. coli DNA repair systems exist, three of which are clearly inducible. 1) Photoreactivation is effected by an enzyme that specifically repairs interstrand cyclobutylthymine dimers induced by far-UV (FUV; 200-300 nm). The photoreactivating enzyme binds to dimers in the dark and, upon exposure to light (ranging from 305-415 nm with peak absorbance at 320, 355, and 380 nm; 4), the covalent bonds between carbons 5 and 6 of the adjacent dimers are eliminated in situ, allowing reformation of normal hydrogen bonding within the DNA double helix. 2) Excision repair is a process that actually removes the lesion from DNA. Resynthesis follows using the complementary, undamaged DNA strand as the template. Initiation of this repair requires an incision step by an enzyme complex specified by the genes uvrA, uvrB, and uvrC (ultraviolet-endonuclease I). Polymerase I recognizes the incision and displaces the damaged strand during the polymerization process. The new patch of DNA is sealed into place by DNA ligase. Kenyon and Walker (5) demonstrated that at least the product of the uvrA gene is inducible (one subunit of the incision enzyme). 3) Recombinational or post-replicational repair is possible because as polymerase III comes to a lesion (e.g., thymine dimer), it stalls for a few seconds and then moves beyond the dimer to reinitiate synthesis. This produces new (or daughter) DNA strands containing gaps that are repaired by sister-strand exchange. These are filled by DNA polymerase I and followed by ligation. Consequently, the DNA area around the replication fork is a composite of old DNA and newly synthesized DNA. This is not because DNA replication is conservative, but because repair is based on single-strand replacement and resynthesis. 4) Error-prone repair is induced by specific DNA-damaging agents. It is not clear if a new polymerase with a reduced proof-reading function is induced, or if the proof-reading function of polymerase III is altered in some fashion. The outcome, however, is that polymerization can occur beyond lesions. This results in the insertion of nitrogenous bases in an apparently random fashion. During the next round of replication, a strand with a new base pair in the area of the original lesion can result (it may be expressed as a mutation, depending on the base inserted as the lesion is by-passed). Although the molecular details of this repair are not fully understood, it appears that this process is responsible for the major fraction of induced mutation. 5) In the broadest sense, the "adaptive response" in E. coli to ethylating and methylating agents can be considered as yet another inducible E. coli repair system. Pretreatment of E. coli with ethylating or methylating agents (e.g., methanesulfonate) results in resistance to the inactivating and mutagenic effects of these agents (6). It has been shown biochemically that at least two enzymes capable of removing alkylated bases are induced in response to alkylating agents (3). The importance of the "adaptive response" with respect to phototoxins is questionable. To our knowledge, no phototoxin has been shown to alkylate DNA. 6) E. coli possesses a series of enzymes that recognize inappropriate nitrogenous bases in DNA and, therefore, are capable of removing

them. This correction system has been described as "mismatch
repair." Occasionally, cytosine deaminates spontaneously yielding
uracil. The enzyme uracil N-glycosylase cleaves the N-glycosylic
bond, attaching the inappropriate base to the DNA backbone. A
second enzyme, apurinic (AP) endonuclease, makes a single-strand
nick 5' to the apurinic or apyrimidic site, allowing for the removal
of a few bases and resynthesis, undoubtedly by polymerase I. Other
glycosylases exist which recognize inappropriate bases in DNA (e.g.,
hypoxanthine N-glycosylase). Although not yet demonstrated, and
perhaps important when considering photosensitized toxic effects, it
has been suggested that glycosylases specific for oxidized nitroge-
nous bases exist (3, 7). 7) Demple and Halbrook (8) recently have
proposed that E. coli has an inducible repair system specific for
oxidative damage independent of those systems just described. Since
the lethality of many phototoxins is based on oxygen-related radi-
cals, such a repair system (or systems) would be particularly impor-
tant to understand. Ultimately, it may turn out that this
postulated system (or systems) represents glycosylases specific for
oxidized nitrogenous bases in DNA. Nevertheless, it has been
reported that at least thirty proteins in E. coli are induced by
"oxidative stress" (9) and it is possible that some of these pro-
teins might be part of the postulated oxidative repair system(s).
        From this brief survey, it is apparent that E. coli exhibits a
multiplicity of systems for coping with DNA damage. Therefore,
using E. coli mutants defective in specific repair capabilities can
provide insights into the mechanism(s) by which the cells respond to
induced mutagenic and lethal lesions. The subject of this paper is
the use of E. coli mutant strains and Haemophilus influenzae trans-
forming DNA (10) to study aspects of the mutagenicity and lethality
of phototoxins.

E. coli RT7h-RT10h — Excision Repair and Catalase (HPII)

Ashwood-Smith et al. (11) were among the first investigators to use
bacterial mutants to aid in characterizing phototoxic effects at the
molecular level. They demonstrated that a FUV-sensitive derivative
of E. coli B/r (B_{s-1}; 12) could be used to quantitatively character-
ize the effects of particular phototoxins (12). Because strain B_{s-1}
is a double mutant (13) in which each mutation contributes in an
additive way to FUV sensitivity, it cannot be deduced from experi-
ments involving this strain which of the two defects carried by the
strain, contributes to its sensitivity to a particular phototoxin.
To circumvent this problem, we developed a series of four E. coli
K12 strains that carry all four possible combinations of genes
controlling excision proficiency (uvrA6 versus $uvrA^+$) and catalase
proficiency (katF versus $katF^+$ [formerly designated as nur versus
nur^+]; 14, 15). The uvrA alleles rather than the recA alleles were
selected for incorporation into these strains because: 1) The uvrA
gene has been shown to be inducible by specific DNA damaging agents
(5) and it is representative of the "SOS system or regulon" (3).
Mutations in the recA gene would eliminate induction of any of the
many components of the SOS regulon, including error-prone repair
necessary for an evaluation of mutagenesis by a particular photo-
toxin. 2) Since recA mutations eliminate reciprocal recombination,
it would not be possible to easily incorporate new genetic markers

into these strains if this proved necessary to extend the investigation of a particular phototoxin in a direction not initially anticipated. The alleles of the uvrA gene permit evaluation of phototoxin-induced DNA lesions reparable by the excision repair system, while the katF alleles allow for evaluation of phototoxin-induced oxidative damage (probably from superoxide anion) since the katF allele sensitizes cells to inactivation by hydrogen peroxide (16).

In addition to the uvrA and katF alleles, we have incorporated a revertible histidine allele (his-4; 17) into these strains. This allows evaluation of mutagenesis by a particular phototoxin in the same experiments designed to determine the nature of the phototoxic lesion(s). These strains (RT7h, RT8h, RT9h, and RT10h, Table I)

Table I. Escherichia coli Strains

Bacterial Strains	Genotype	Reference or Source
RT7h(tet^r)	F$^-$, thy-1, argA1, lysA22 mtl-2, malA1, str-104 λ^r λ^-, supE44?	14
RT8h(tet^r)	same as RT7h(tet^r) except thi$^+$, uvrA$^+$	
RT9h(tet^r)	same as RT7h(tet^r) except nur$^+$	
RT10h(tet^r)	same as RT7h(tet^r) except thi$^+$	
GW1060	F$^-$, thr-1, leu-6, his-4, argE3, ilvts, tif, sfiA11, Δlac(U169), galk2, str31, uvrA215::Mud(Aplac)	5
K1060	F$^-$, fadE62, IacI60, mel-1, supE57, supF58	27, 30

have been tested with phototoxins with known mechanisms of action (18, 19), such as psoralen whose phototoxicity is almost exclusively due to cycloadditions to DNA. When the four strains were treated with psoralen plus near-UV (NUV; 300-400 nm), strains RT7h and RT9h which carried the uvrA6 allele proved to be sensitive to inactivation (Figure 1). This result suggests that psoralen lesions (cycloadditions to DNA) are reparable by the excision-repair system. The katF allele (formerly designated nur) does not sensitize strains to psoralen plus NUV. This can be interpreted as evidence that oxygen-related radicals are not formed upon NUV treatment in the presence of psoralen and is consistent with the chemical experiments involving psoralen plus NUV. Furthermore, in these same experiments, we demonstrated, as expected, that psoralen plus NUV treatment is mutagenic, although it is not as efficient a mutagen as is FUV alone (19).

When the four strains were treated with alpha-terthienyl (α-T), the kinetics of inactivation were indistinguishable (Figure 2). In addition, histidine-independent mutants were undetectable. Available evidence suggests that α-T acts as an oxygen-dependent
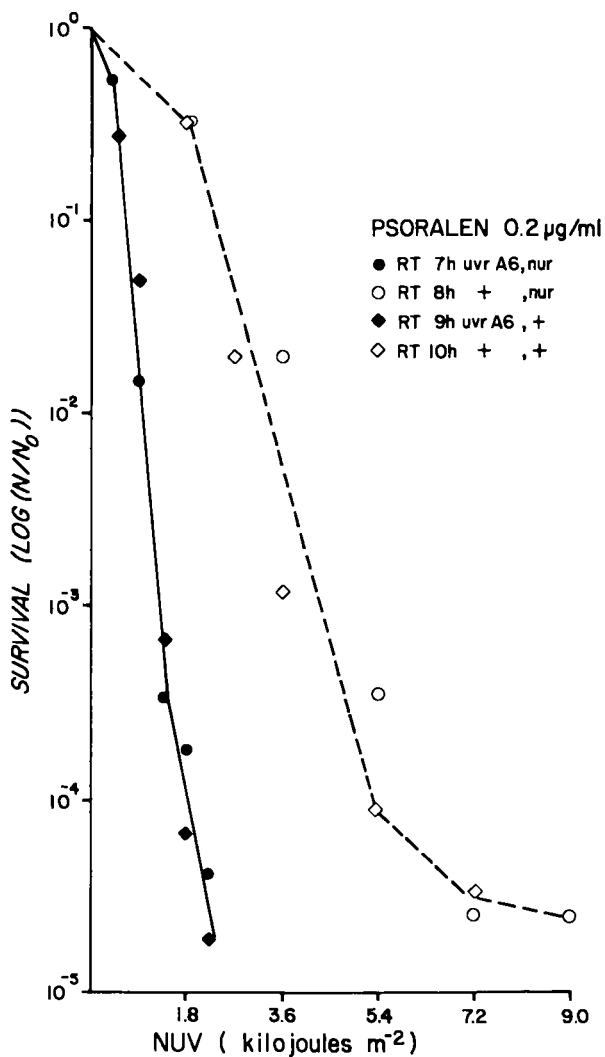
Figure 1.   Fluence–response curve for four E. coli strains treated with broad–spectrum NUV in the presence of psoralen.   (Reproduced with permission from Reference 19.   Copyright 1986 Plenum.)
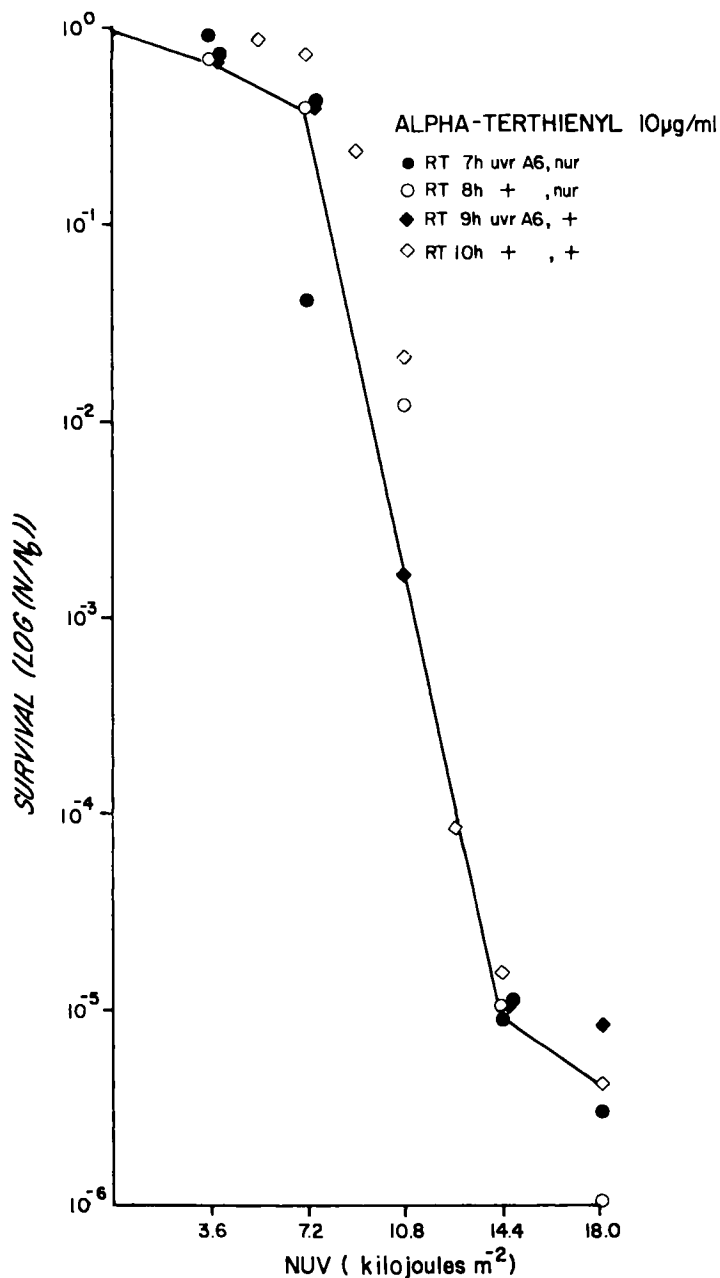
Figure 2. Fluence—response curves for four E. coli strains treated with broad—spectrum NUV in the presence of α—terthienyl. (Reproduced with permission from Reference 19. Copyright 1986 Plenum.)

(photodynamic) photosensitizer (20) with the principal target being
the membrane (21) and our results are consistent with these conclu-
sions (Figure 2). The inactivation kinetics for the four strains
are indistinguishable and implies that the uvrA and katF alleles do
not influence the sensitivity of the cells to inactivation by α-T
plus NUV. This is exactly what might be anticipated if α-T did not
enter the cell to generate oxygen radicals which are capable of
damaging DNA or other cellular components in the cytosol. One would
expect α-T plus near-UV to be non-mutagenic if penetration into the
cell does not occur. The half-life of singlet oxygen ($^1O_2$) is
extended in non-polar environments (22) and the inactivation of the
four strains may be caused by this reactive oxygen species that
damages either the proteins or the fatty acid components of the
membrane (see the section entitled: E. coli Strain K1060 -- Fatty
Acid Auxotroph).
        In collaboration with J. Kagan, we used the four E. coli
strains (RT7h-RT10h) to evaluate the phototoxicity of a non-carcino-
genic, polycyclic aromatic hydrocarbon (PAH), fluoranthene. We
found that fluoranthene plus NUV causes damage to DNA because the
strains carrying the uvrA6 mutation were sensitive to inactivation.
In addition, the katF allele sensitized the cells to inactivation by
fluoranthene plus NUV, which implied that fluoranthene plus NUV
generates superoxide anion, leading to H_2O_2 and ultimately to the
hydroxyl radical (23). Chemical experiments have confirmed these
conclusions (manuscript submitted). We also showed that fluor-
anthene plus NUV treatment did not induce histidine-independent
mutations which suggested that the treatment did not induce the
error-prone repair system known to be part of the SOS regulon (3).
Since the uvrA gene is part of this same regulon, we predicted that
treatment with fluoranthene plus NUV would not induce the uvrA gene
product (see section entitled: E. coli Strain GW1060 -- Induction
of the uvrA Gene Product).
        The experiments described above illustrate how the four genet-
ically defined E. coli K12 strains (RT7h-RT10h) can be used to draw
tentative conclusions about the mechanism(s) of mutagenesis and
lethality by particular phototoxins. These conclusions may be used
as guides for additional chemical and biological experiments.

E. coli Strain GW1060 -- Induction of the uvrA Gene Product

Operon fusion strains provide an efficient system for studying the
regulation of loci whose gene products are unknown or difficult to
assay. In particular, the Mud(Aplac) bacteriophage, with its appar-
ently random integration and easily assayed gene product, has been
extremely useful in these studies of gene regulation (24). Of the
various Mud(Aplac) insertions, GW1060, an E. coli strain with a
Mud(Aplac) insertion in the uvrA locus [uvrA215::Mud(Aplac)], can be
used to demonstrate that the expression of the uvrA gene is
inducible by DNA-damaging agents such as FUV and mitomycin C (5;
Figure 3A). In this fusion strain, β-galactosidase expression is
induced by these DNA-damaging agents in a $recA^+$ lex^+-dependent
fashion (5, 25).
        We have tested three phototoxins for their ability to induce
the uvrA gene product using strain GW1060. To establish that the
strain behaves in our hands as it did for Kenyon and Walker (5), we

treated the cell with FUV and mitomycin C. The cells behaved
exactly as described previously (Figure 3A; 5). We anticipated that
psoralen plus NUV should be capable of inducing the uvrA gene prod-
uct since psoralen has been shown to form cycloadducts to DNA. The
results displayed in Figure 3C substantiate this prediction. The
induction is clearly the result of the combination of psoralen plus
NUV since neither psoralen nor NUV alone (Figure 3B and C) induce
the uvrA gene product.

        Reported data suggest that α-T does not enter cells, but causes
lethal damage to the membrane (20, 21). Our results are totally
consistent with α-T operating at the membrane level (19: see sec-
tion entitled: E. coli Strains RT7h-RT10h -- Excision Repair and
Catalase (HPII)). We predicted correctly that α-T plus NUV would
not induce the uvrA gene product if the membrane were the lethal
target (Figure 3C). Induction of the uvrA gene product was not
observed with fluoranthene, a non-carcinogenic PAH plus NUV (Figure
3C). This seems paradoxical at first glance since the uvrA6 allele
sensitized strains RT7h and RT9h to inactivation by fluoranthene
plus NUV. It is consistent, however, with the observation that
fluoranthene plus NUV is not capable of inducing histidine-independ-
ent mutations. Thus our interpretation is that fluoranthene plus
NUV produces damage to DNA and the uninduced level of the uvrA gene
product can repair these lesions. Alternatively, the damage pro-
duced by fluoranthene plus near-UV does not form an inducing signal
necessary to increase levels of the uvrA gene product as well as
allowing formation of the error-prone repair system which leads to
histidine-independent mutations. From the GW1060 results, one can
suggest that α-T plus NUV induces damage that is oxidative since the
results with α-T plus NUV parallel those obtained with H_2O_2. Hydro-
gen peroxide does not induce histidine-independent mutants in
strains RT7h-RT10h (7) and it does not induce the uvrA gene product
(Figure 3B).

        In conjunction with the inactivation experiments with strains
RT7h-RT10h, strain GW1060 can be used to establish whether a photo-
toxin is capable of producing a signal for the induction of at least
one component of the SOS regulon (uvrA gene product). The results
from these two entirely different experimental systems can be viewed
as complementary and confirmatory (e.g., α-T plus NUV is not muta-
genic [for the his-4 locus at least] and it does not induce either
the uvrA gene product or the error-prone repair system, a portion of
the SOS regulon necessary for mutation). The results from the
experiments with GW1060 and fluoranthene plus NUV parallel the
results obtained when GW1060 and H_2O_2 were used. This implies that
superoxide anion is an important product of fluoranthene plus NUV
treatment. The chemical results confirmed this expectation (manu-
script submitted).

E. coli K1060 -- Fatty Acid Auxotroph

E. coli strain K1060 can neither synthesize nor degrade unsaturated
fatty acids (fatty acid auxotroph). Therefore, it is possible to
control the amount and type of unsaturated fatty acid in the mem-
brane by growing the cells in medium of a particular composition
supplemented with the appropriate fatty acid (26, 27). In addition
to DNA and soluble proteins, phototoxins (e.g., α-T; 21) may attack
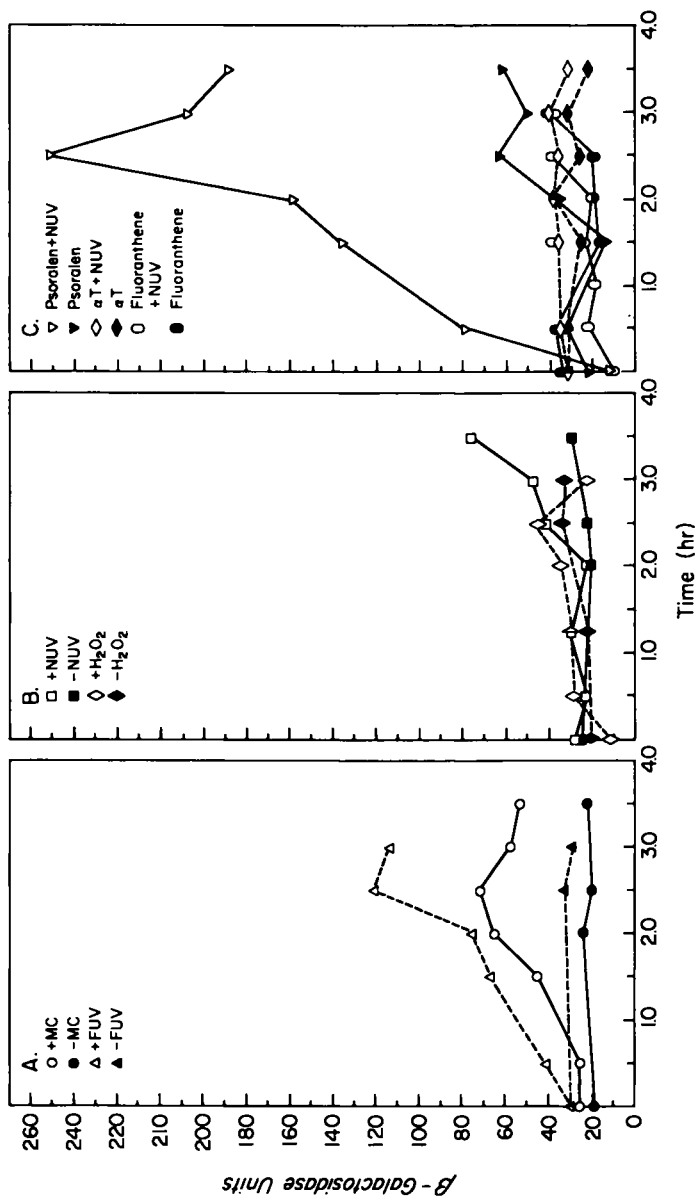
Figure 3. Beta-galactosidase activity of E. coli cells (strain GW1060) following treatment with various possible inducing agents.

the proteins or the fatty acids of the membrane. Because the half-
life of $^1O_2$ is extended in non-polar environments ($\underline{22}$) and because
$^1O_2$ reacts with unsaturated fatty acid double bonds ($\underline{28}$, $\underline{29}$), oxy-
gen-dependent phototoxins that do not have DNA as an important
lethal target probably generate $^1O_2$ that reacts with the unsaturated
fatty acids of the membrane destroying its hydrophobicity leading to
generalized membrane disintegration. If this is true, we should be
able to test this possibility by growing $\underline{E. coli}$ strain K1060 in the
presence of fatty acids with varying degrees of unsaturation. The
practicality of this approach was illustrated by experiments with
strain K1060 that was grown first with twenty carbon fatty acids
(eicosaenoic acid derivatives) exhibiting various degrees of unsatu-
ration (1, 2, 3, or 4) and then treated with NUV. These experiments
showed that the sensitivity to inactivation by NUV of the various
exponentially growing populations was related to the degree of
unsaturation of the fatty acids used for supplementation. The
greater the degree of unsaturation, the greater the sensitivity of
the cells to inactivation by NUV ($\underline{30}$). We have shown that important
endogenous photosensitizers for NUV in $\underline{E. coli}$ cells are the cyto-
chromes located within the membrane ($\underline{15}$). It seems likely that
absorption of NUV wavelengths by cytochromes generates $^1O_2$ which has
the potential for reacting with the unsaturated fatty acids within
the membrane. This would account for the lethality and relative
non-mutability of these wavelengths. If the results reported by
Downum $\underline{et}$ $\underline{al}$. ($\underline{21}$) suggesting that the lethal targets for inactiva-
tion by α-T plus NUV are membrane proteins are correct, cells of
strain K1060 grown with fatty acids having various degrees of unsa-
turation should be equivalent in their sensitivity to inactivation
by α-T plus NUV. The results of experiments designed to test this
prediction are presented in Figure 4. If the fatty acids of the
membrane were an important lethal target, strain K1060 grown with
arachidonic acid (20°C, 4 double bonds) supplementation should have
been sensitive to inactivation by α-T plus NUV. Clearly, these
cells were no more sensitive to inactivation by α-T plus NUV than
were cells grown with 11-eicosaenoic acid supplementation (20°C, 1
double bond). We conclude from these results that the lethality of
α-T plus NUV to $\underline{E. coli}$ cells results from damage to membrane pro-
teins, as suggested by Downum $\underline{et}$ $\underline{al}$. ($\underline{21}$). It should be noted that
the NUV fluences used in these experiments do not result in signifi-
cant inactivation unless α-T is present.
      Strain K1060 can be used with phototoxins, the lethal target of
which appears to be the membrane. It can be used to differentiate
between the unsaturated fatty acids of the membrane and the essen-
tial membrane proteins as the lethal targets.

E. coli HB101 -- Cloned Carotenoid Genes

Recently, Professor Jeffrey L. Bennetzen's group (Department of
Biology, Purdue University) has cloned carotenoid genes from $\underline{E.}$
$\underline{stewartii}$ (the incitant of corn wilt) into $\underline{E. coli}$ strain HB101, and
they have obtained expression of these genes. Professor Bennetzen
has provided us with the strains in which carotenoids are expressed
and the HB101 strain into which the carotenoid genes were cloned.
Since carotenoids are part of the membrane, the carotenoid-producing
$\underline{E. coli}$ strains can be used to test whether carotenoids protect the
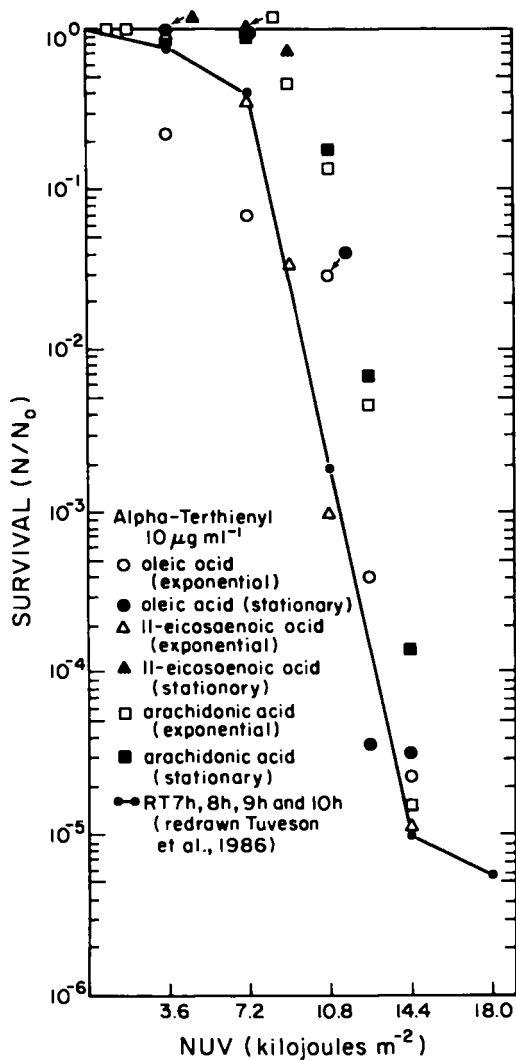
Figure 4. Fluence-response curves for exponentially growing and stationary cell populations of E. coli strain K1060 when grown with various fatty acids as supplements.

cell against inactivation by a particular phototoxin. If protection
is observed, one could postulate that the principal lethal events
occur within the membrane and that singlet oxygen may be involved.
These results would complement those obtained with E. coli strain
K1060 (fatty acid auxotroph). Although investigations with the
carotenoid and carotenoidless E. coli strains are in the early
stages, we have shown that the carotenoid-producing strains are
protected against the inactivating effects of a β-carboline alkaloid
(harmine) plus NUV as well as NUV alone. The carotenoid and carote-
noidless E. coli strains hold great promise as possible tools to aid
in the biological investigations of phototoxins.

Transforming DNA -- Haemophilus influenzae

Transforming DNA is a relatively simple and direct method for
testing the effects of phototoxins on the biological activity of
DNA, and transforming DNA from Haemophilus influenzae (10, 31) is
the system of choice. Cells can easily be made competent by using a
brief period of anaerobiosis and can be stored at -80°C for months
with only a modest loss in competence. With little difficulty,
transforming DNA from whole cells can be prepared by standard proce-
dures. Platings to assay for transformation can be done by pour
plating since this H. influenzae is not an obligate aerobe, as is
Bacillus subtilis. In addition, pour-plating permits counting hun-
dreds of colonies with little difficulty.
     We have used H. influenzae transforming DNA to test the effi-
cacy of various phototoxins for the inactivation of transforming
activity. The general result is that phototoxins that form cycload-
ditions to DNA (e.g., psoralen and angelicin) are highly efficient
for the inactivation of transforming activity. Phototoxins that
appear to act chiefly by the generation of oxygen radical species
(α-T and fluoranthene) are much less efficient. When comparing
psoralen (0.2 μg ml^{-1}) with fluoranthene (10.0 μg ml^{-1}), for exam-
ple, the fluence required to reduce transforming activity to 0.37
was 15-fold greater with fluoranthene (manuscript submitted). Our
impression is that the phototoxins we have investigated separate
into two broad categories with respect to inactivation of trans-
forming activity, those that covalently bind to DNA, and those that
form oxygen-related radicals. The latter are relatively ineffective
in reducing transforming activity and seem to be strongly influenced
by the solvent in which the phototoxin is dissolved (presumably
reflecting excimer formation). We are currently testing this possi-
bility.

Conclusions

The biological activity of suspected phototoxins can be assessed in
a matter of days (probably two working weeks once the systems are in
place) by using the bacterial systems described in this paper.
Microbial systems are easy to use and results can be obtained
quickly. These biological experiments, in conjunction with the
appropriate chemical experiments, can result in an accurate picture
of the mode of action of a particular phototoxin before the much
more laborious tests involving eucaryotic biological systems are
undertaken. Determining the mechanism of action of a phototoxin

using microbial systems provides plausible explanations for results
obtained with eucaryotic systems in which mutants are usually not
available.

Acknowledgments

Literature Cited

1. Bachman, B. J. Microbiol. Rev. 1983, 47, 180–230.
2. Witkin, E. M. Bacteriol. Rev. 1976, 40, 869–907.
3. Walker, G. C. Microbiol. Rev. 1984, 48, 60–93.
4. Jagger, J. Photochem. Photobiol. 1964, 3, 451–61.
5. Kenyon, C. J.; Walker, G. C. Nature (London) 1981, 289, 808–
    10.
6. Samson, L.; Carins, J. Nature (London) 1977, 267, 281–2.
7. Sammartano, L. J.; Tuveson, R. W. Photochem. Photobiol. 1985,
    41, 367–70.
8. Demple, B.; Halbrook, J. Nature (London) 1983, 30, 466–8.
9. Christman, M. F.; Morgan, R. W.; Jacobson, F. S.; Ames, B. N.
    Cell 1985, 41, 753–62.
10. Setlow, J. K.; Brown, D. C.; Boling, M. E.; Mattingly, A.;
    Gordon, M. P. J. Bact. 1968, 95, 546–58.
11. Ashwood-Smith, M. J.; Poulton, G. A.; Ceska, O.; Lieu, M.;
    Furniss, E. Photochem. Photobiol. 1983, 38, 113–8.
12. Hill, R. F.; Simson, E. J. Gen. Microbiol. 1961, 24, 1–14.
13. Greenberg, J. Genetics 1967, 55, 193–201.
14. Leonardo, J. M.; Reynolds, P. R.; Tuveson, R. W. Mutat. Res.
    1984, 126, 1–8.
15. Tuveson, R. W.; Sammartano, L. J. Photochem. Photobiol. 1986,
    43, 621–6.
16. Sammartano, L. J.; Tuveson, R. W.; Davenport, R. J. Bact.
    1986, 168, 13–21.
17. Kato, T.; Rothman, R. H.; Clark, A. J. Genetics 1977, 87, 1–
    18.
18. Scott, B. R.; Pathak, M. A.; Mohn, G. R. Mutat. Res. 1976, 39,
    29–74.
19. Tuveson, R. W.; Berenbaum, M. R.; Heininger, E. E. J. Chem.
    Ecol. 1986, 12, 933–48.
20. Arnason, T.; Chang, G. F. Q.; Wat, C. K.; Downum, K.; Towers,
    G. H. N. Photochem. Photobiol. 1981, 38, 811–24.
21. Downum, K. R.; Hancock, R. W. E.; Towers, G. H. N. Photochem.
    Photobiol. 1982, 36, 517–23.
22. Korycka-Dahl, M. B.; Richardson, T. In CRC Critical Reviews
    in Food Science and Nutrition; Furia, T. E., Ed.; CRC Press,
    Inc.: West Palm Beach, FL, 1978; pp 209–38.
23. Larson, R. A. J. Chem. Ecol. 1986, 12, 859–70.
24. Casadaban, M. J.; Cohen, S. N. Proc. Natl. Acad. Sci. (USA)
    1979, 76, 4530–33.
25. Kenyon, C. J.; Walker, G. C. Proc. Natl. Acad. Sci. (USA)
    1980, 77, 2819–23.

26. Taylor, F.; Cronan, J. E. J. Bact. 1976, 125, 518-23.
27. Redpath, J. L.; Patterson, L. K. Radiat. Res. 1978, 75, 443-7.
28. Hall, G. E.; Roberts, D. G. J. Chem. Soc. (B) 1966, 11, 1109-12.
29. Cobern, D.; Hobbs, J. S.; Lucas, R. A.; MacKenzie, D. J. J. Chem. Soc. (C) 1966, 21, 1897-1902.
30. Klamen, D. L.; Tuveson, R. W. Photochem. Photobiol. 1982, 35, 167-73.
31. Tuveson, R. W. Genet. Res. (Camb.) 1972, 20, 9-18.

Chapter 14

Charge of the Light Brigade:
Phototoxicity as a Defense Against Insects

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Sunlight is used by many plants to activate secondary
compounds and to enhance their toxicity. This
activation can occur in at least two ways. Photons can
be absorbed by plant chemicals, such as the
furanocoumarins typical of the Umbelliferae and
Rutaceae, to alter the electron configuration and form
a highly reactive excited state; the excited state
molecule can then interact directly with biomolecules
such as DNA, proteins or membrane lipids with
concomitant toxic effects. Alternatively, as is the
case for polyacetylenes typical of the Compositae and
quinones of the Guttiferae, photopromoted excited
states can interact with oxygen to form the reactive
molecule singlet oxygen, which then can interfere
chemically with other biomolecules. Toxicity
enhancement by sunlight is increased still further by
virtue of the fact that certain wavelengths can
stimulate enhanced biosynthesis and increased
accumulation of phototoxins. Naturally occurring
phototoxins occur in a diverse array of plant families
and represent a variety of biosynthetically unrelated
structures. Many of these chemicals are toxic to
generalized feeders , particularly in the presence of
light of the appropriate wavelengths. Essentially
every phototoxic plant is associated with oligophagous
species which have overcome the defensive chemistry of
their hosts. Mechanisms of resistance include
behavioral resistance in the form of leaf-rolling, web-
spinning, and other forms of concealed feeding which
shield the insect from damaging wavelengths, physical
resistance in the form of body pigments that
selectively absorb damaging wavelengths or quench
excited states, or biochemical resistance in the form
of enzymatic degradation of phototoxic molecules.
Sunlight, then, is an important ecological factor
mediating the evolutionary responses between plants and
herbivorous insects.

The concept of using light energy for defensive purposes, (viz, the "Strategic Defense Initiative" of the Reagan administration) is hardly an innovation; plants have incorporated sunlight into their defensive armamentarium for millenia (1). Many plant allelochemicals can absorb photons of light energy at particular wavelengths. This energy can transform a molecule from its lowest electron energy state or ground state to a higher or excited state. These excited states are highly reactive and phototoxic plant chemicals can react with a variety of biomolecules. In the case of furanocoumarins, for example, compounds typically found in plants in the families Rutaceae and Umbelliferae, excited triplets react with pyrimidine bases in DNA to form cycloadducts that impair transcription and replication. In many other photosensitizers, the excited triplet state react with molecular oxygen, which in its ground state is a triplet. The singlet oxygen that results is highly reactive and can damage proteins, lipids and DNA. Ground state oxygen can also form superoxide radicals in the presence of a photosensitizer; these molecules can damage lipids, DNA, and polysaccharides (1).

Since the target sites for photosensitizing compounds are often important biomolecules, natural photosensitizers are broadly biocidal. However, it has long been recognized (2) that herbivorous insects, as a major selective force on plants, are likely to be a principal motive force behind the evolutionary proliferation of toxic chemicals in plant tissue. Such is likely the case for photosensitizers as well. Natural phototoxins were first shown to have insecticidal properties in 1978 (3); since that time, at least nine biosynthetically distinct classes of phototoxic insecticides have been identified (Table I). Sunlight, then, can act at the chemical level, enhancing the toxicity of defensive chemicals synthesized by plants. Sunlight can also affect metabolic rates in plants; increasing UV light intensity selectively stimulates enzyme activity and enhances biosynthetic rates for a variety of natural products (Table II). Induction of phenylalanine ammonia lyase by ultraviolet irradiation, e.g., directly affects production of phenylpropanoids, coumarins, flavonoids, acetophenones and lignans (Berenbaum 1987, in press). Moreover, sunlight can act as an indirect factor influencing the chemical profile of a plant species. In that wavelength and intensity are two factors influencing photosynthetic rates of plants, they can also influence allelochemical production in those instances in which biosynthesis is energy-limited. Enhanced photosynthetic rates provide more energy to channel into biosynthesis (4).

Table I. Plant derived-phototoxins with insecticidal properties

Class	Source	Reference
Acetylenes	Compositae	5, 6, 7
Benzopyrans and furans	Compositae	Aregullin, this volume
Benzylisoquinoline alkaloids	Berberidaceae, Rutaceae, Rubiaceae	8
Beta-carboline alkaloids	Rutaceae, Simaroubaceae	9, E. Heininger, in prep.
Extended quinones	Guttiferae, Polygonaceae	10
Furanocoumarins	Leguminosae, Moraceae, Rutaceae, Umbelliferae, Composite Solanaceae	3, 11, 12, 13, 14
Furanochromones	Rutaceae, Umbelliferae	11, 15
Furoquinoline alkaloids	Rutaceae	9, E. Heininger, in prep.
Thiophenes	Compositae	5, 7, 16, 17

Table II. Plant compounds induced or increased by light

Plant compound	Light source	Plant source	Ref
Alkaloids	red and IR	tobacco	18
Alkaloids	Visible	lupines,tobacco	18
Anthocyanins	Visible	many plants	19
Betacyanins	red	Centrospermae	19
Cannabinoids	UV	marijuana	20
Cardenolides	Visible	Digitalis lanata	21
Carotenoids	blue light	many species	22
DIMBOA	Visible	Zea mays	23
Flavonoids	UV	Umbelliferae	24
Furanocoumarins	UV	parsnip	Berenbaum and Zangerl 1987, in press
Isoflavonoids	UV	soybean	25
Tannins	"sunlight"	oak	26
Terpenes	"sunlight"	Hymenaea courbaril	27

Efficacy of phototoxicity as a defense against insects

No defense system is unbreachable, and light-dependent defense systems of plants are no exception. Continuing selection by plant chemicals promotes the acquisition of resistance by herbivorous insects over evolutionary time. However, selection by phototoxins differs from the standard scenario (e.g., 28) in that the selective force promoting the evolution of resistance can either be the chemical itself or the sunlight conferring toxicity to the chemical. Insect associates of phototoxic plants display a variety of adaptations to phototoxic plants that either reduce the chemical reactivity or physiological effects of the substances involved or minimize their exposure to lethal amounts or wavelengths of sunlight.

Behavioral resistance

Behavioral resistance to a phototoxin results either from the failure of an insect to ingest or contact a lethal dose of toxicant or from the ability of an insect to feed in such a manner as to reduce the amount of light exposure below that required to activate a phototoxin. Feeding in a concealed manner is characteristic of a number of insect associates of a number of phototoxic plants. Modes of concealed feeding include leaf mining, leaf tying, leaf rolling, stem boring, subterranean root feeding, or boring into buds or fruits. In all these cases, plant tissues can effectively block significant amounts of damaging sunlight. For example, in a survey of leaf epidermal transmittance of UV radiation in 25 species of plants, transmittance in most cases was less than 10% and in over half the species ranged from 1 to 5% (29). Behavioral avoidance of phototoxins is a widespread phenomenon; over 70% of the fauna of phototoxic Umbelliferae in one study consisted of insects feeding in a concealed manner (3). Concealed feeders can either be highly specialized (as is the case for umbellifer-feeding leaf-mining Agromyzidae) or broadly polyphagous (as is the case for Choristoneura rosaceana, a tortricid leafroller that feeds on a number of phototoxic plants in the Umbelliferae and in several other families). Champagne et al. 1986 suggest that, in addition to boring into stems, spinning profuse amounts of silk also serves to attenuate incoming radiation and to protect Ostrinia nubilalis, the European corn borer, from phototoxic acetylenes in its hosts in the family Compositae.
        The most compelling evidence for a photoprotective role for concealed feeding involves the insect fauna of Hypericum perforatum, St. Johnswort or Klamath weed. H. perforatum contains an extended quinone pigment, hypericin, which is activated by sunlight in the region of 500-600 nm (30). Although a proportion of the fauna of St. Johnswort consists of specialists, there are several broadly polyphagous species that can predictably be found feeding on the foliage and flowers (Table III). Of these, one species (a lycaenid caterpillar) bores into flowers and fruits, and five (all tortricid caterpillars) tie together leaves, stems or flowers and feed inside these ties. Ostensibly, since foliage is essentially opaque to most wavelengths of light, caterpillars concealed in leaf ties can feed

on phototoxic material with impunity; insufficient amounts of light penetrate to activate the phototoxin. When one of the tortricids, Platynota flavedana, is reared in the laboratory on an artificial diet, it cannot engage in leaf-tying behavior and is forced to feed in an manner such that it is exposed to light. Caterpillars reared on an artificial diet containing hypericin suffered significantly greater mortality when exposed to full sunlight than when they were protected from damaging wavelengths by an acetate filter (Table IV - S. Sandberg, in prep.). Leaf-tying may thus be a preadaptation allowing generalized feeders, lacking a specific detoxication system for phototoxins, to exploit phototoxic plants. Such behavior may in fact be facultative, when P. flavedana feeds on the foliage of strawberry (Fragaria virginiensis), a nonphototoxic plant, it occasionally spins only a scaffolding of silk in place of a leaf fold in its early instars. Of the remaining lepidopterous associates of Hypericum, species in the noctuid genus Polia feed nocturnally (G. Godfrey, pers. comm.), when risks of phototoxicity are minimized. Nocturnally active insects in general may be preadapted for feeding on phototoxic plants.

Table III. Lepidopteran associates of Hypericum

Species	Family	Mode of feeding	Host range	Ref
Strymon melinus	Lycaenidae	flower/fruit borer	Generalist	31
Zale lunata	Noctuidae	external folivore	Generalist	31
Polia assimilis	Noctuidae	external folivore	Salicaeae, Compositae Guttiferae	31
Delta ramosula	Noctuidae	external folivore	Guttiferae	31
Delta stewarti	Noctuidae	external folivore	Guttiferae	31
Hyperetis amicaria	Geometridae	external folivore	Generalist	31
Eupithecia miserulata	Geometridae	external folivore	Generalist	Sandberg, in prep.
Pleuroprucha insularia	Geometridae	external folivore	Generalist	Sandberg, in prep.
Platynota flavedana	Tortricidae	leaf tyer	Generalist	Sandberg, in prep.
Sparganothis sulfureana	Tortricidae	leaf tyer	Generalist	Sandberg, in prep.
Xenotemna pallorana	Tortricidae	leaf tyer	Generalist	Sandberg, in prep.
Choristoneura parallela	Tortricidae	leaf folder	Generalist	Sandberg, in prep.
Unidentified sp.	Gracillariidae	leaf folder		Sandberg, in prep.

Table IV. Effects of hypericin on Platynota flavedana in the presence and absence of light (S. Sandberg, in preparation)

4a. Survivorship (%) of Platynota flavedana to second instar (n = 40 in each treatment)'

	Full light	Filtered light
Control diet	80.0	85.0
0.03% hypericin diet	50.0	77.5

' A G^2 test of independence yielded a value of .109 for the interaction of hypericin and light regime, indicating the toxicity of hypericin is affected by the light regime

Resistance to phototoxins

Many insects may rely on physical factors for protection from
phototoxins; in these cases, although the insect ingests or contacts
phototoxins and is exposed to light, the light fails to reach the
target site of the molecule. In mammals, dark-skinned individuals
are relatively more immune to the effects of ingestion of or contact
with phototoxins (32). This resistance is attributable to the
differential concentrations of melanin. Melanin acts as a
photoprotective agent in several ways. Melanin absorbs both UV and
visible light and acts as a neutral density filter; melanin-
containing melanosomes scatter incoming radiation and attenuate the
light; melanin can absorb radiant energy and dissipate it as heat;
it can also, as a stable free radical, act as a "biologic electron
exchange polymer" (32). Although much of the brown or black
coloration of insect cuticle is attributable to tanning (i.e., the
protein-quinone crosslinkage involved in sclerotization), dark
coloration in many species is due to deposition of melanin (33). At
least two species of insects associated with plants containing
phototoxins are prone to melanic mutations. Melanic larvae of
Papilio machaon (the Old World swallowtail), an associate of
phototoxic Umbelliferae, are known to occur (34). Manduca sexta,
the tobacco hornworm (Lepidoptera: Sphingidae), feeds on the foliage
of Solanaceae, including Lycopersicon esculentum, the tomato, which
is reported to contain the phototoxic furanocoumarin bergapten (35).
A mutant form arises on occasion in which the normally transparent
cuticle turns black in the ultimate larval instar due to hormonally
mediated pigment deposition (36). These individuals can comprise up
to 10% of natural populations (G. Kennedy, personal communication
1986). The phototoxic furanocoumarin xanthotoxin was topically
applied in acetone at the rate of 50 micrograms/g body weight to the
dorsal area of the thorax of ultimate instar caterpillars with
normal pigmentation. This treatment in the presence of UV light
resulted in major injury to the pupae. Specifically, pupal wings
failed to form and to sclerotize properly. Seventy percent of the
treated individuals failed to pupate at all or manifested cuticular
damage to wings. That the damage was essentially limited to the
developing wings is consist with the interpretation that mitotically
active tissue (such as developing wing imaginal discs) is
particularly susceptible to the antimitotic effects of irradiated
furanocoumarins. When black mutant hornworm larvae were treated in
an identical fashion in the ultimate instar, only 30% failed to
pupate or exhibited wing deformities (Wiseman and Berenbaum, in
preparation). Melanin, then, appears to confer protection against
the photoactivation of furanocoumarins by UV light and such
protection may account for the persistent presence of melanic
individuals in some insect populations.
      Highly reflective surfaces may also confer some protection
against phototoxins (37). Several species of chrysomelid beetles
are frequent associates of the genus Hypericum; of these, species in
the genus Chrysolina are characteristically metallic blue-black in
color. Their highly reflective surface may prevent visible light
from entering the body cavity to activate ingested hypericin-
containing plant tissue (38). In general metallic colors, while

absorbing incident radiation well, absorb poorly. The metallic
cuticle of tiger beetles reflect substantial amounts of shortwave
radiation, ranging from 280 to 580 nm (39).

Biochemical resistance to phototoxins

Biochemical resistance to phototoxins has been documented in several
insects associated with phototoxic plants. Biochemical resistance
involves metabolism of a toxin such that it is no longer toxic.
     One general biochemical defense against phototoxins is to
intercept a photoactive molecule with another molecule to maintain
it in the ostensibly nontoxic ground state (38). Larson 1986
suggested that many insects produce or sequester chemicals with the
ability to physically "quench" excited states—that is, to remove
energy from an excited state "donor" molecule without undergoing
structural change. Beta carotene and related carotenoids, which are
excellent quenchers, are widespread in the hemolymph, wings and
integument of herbivorous insects (33, 40) and may function as
quenchers for those species feeding on phototoxic plants. In
addition, due to its absorption maxima (around 450 to 550 nm), beta
carotene may directly quench the phototoxic furanocoumarins, which
show maximal fluorescence in that region. Nitrogen-containing
pigments such as the pterines and the ommochromes may also be
involved in oxygen quenching, inasmuch as similar alkaloids possess
this property (41).
     Toxic oxygen species are also subject to physical and
biochemical detoxication in insects that feed on phototoxic plants.
Certain insect constituents of cuticle or hemolymph are, or
resemble, structurally efficient quenchers of singlet oxygen. These
include carotenoids, amines, and sulfur and oxygen derivatives (38).
Flavonoid pigments can act as efficient singlet oxygen scavengers as
well. Quercitin, a widely distributed plant constituent, can
suppress singlet-oxygen dependent reactions (42). Glycosides of
quercitin appear to be selectively sequestered from their foodplants
by swallowtail butterflies in the tribe Graphiini and by one species
in the genus Papilio (43). Phototoxic alkaloids (e.g., berberine)
are reported to occur in the annonaceous hosts of these butterflies
(43) and the umbelliferous hostplants of Papilio machaon, the
species sequestering quercitin glycosides, are known to contain
furanocoumarins, several of which generate singlet oxygen in the
presence of UV (44).
     Other species of swallowtails, particularly in the Troidini and
Papilionini, selectively sequester carotenoids; overall the
concentration of carotenoids in Papilionidae is up to an order of
magnitude higher than concentrations in other butterfly families
(Table V). Rothschild et al. (45) suggested that carotenoid
sequestration may serve to protect troidines associated with
Aristolochiaceae by preventing free-radical oxidation of the
nitrophenanthrene aristolochic acids to phenolics. In addition,
carotenoids may serve as singlet oxygen quenchers for the several
classes of photosensitizers (including furanocoumarins,
furoquinoline alkaloids, furochromones, and benzylisoquinoline
alkaloids) present in the Rutaceae, principal host family for the
majority of papilionines (46).

Table V. Carotenoid content of butterflies (40)

Family	Total ug/g dry weight
Lycaenidae	79.4
Nymphalidae	62.1
Pieridae	32.7
Satyridae	70.4
Papilionidae*	297

* Calculated from Rothschild (45), Valadon and Mummery (47)

Specific biochemical pathways for detoxification are known to exist in some species of insects adapted to feeding on phototoxic plants. Ivie and colleagues (48-49) have extensively documented the mixed-function oxidase-mediated detoxification of furanocoumarins by the black swallowtail Papilio polyxenes. Alternate biochemical detoxification systems may exist as well. Depressaria pastinacella, the parsnip webworm, is an oecophorid caterpillar that feeds exclusively on the wild parsnip, Pastinaca sativa, which contains several phototoxic furanocoumarins (50). Toxicity of furanocoumarins is not enhanced by mixed function oxidase inhibitors such as the methylenedioxyphenyl-containing myristicin (J. Nitao, in preparation); this observation suggests that an alternate route is in force. In fact, substantial amounts of orally administered xanthotoxin (a furanocoumarin found in the parsnip hostplant) are recoverable intact in the silk and silk glands of the caterpillar, raising the possibility that the parsnip webworm sequesters plant-derived phototoxins for its own defense when ensconced in larval webbing or pupal cocoon silk (J. Nitao, in preparation).

Ecological variation

To a great extent, ecological factors can influence the efficacy of phototoxicity as a defense against insects. On a very small scale, shade availability may determine the relative susceptibility of individual plants in a population to insects. For example, wild parsnips grown under conditions of 50 or 70% ambient light show a significant reduction in furanocoumarin content of the foliage (Berenbaum and Zangerl 1986); inasmuch as furanocoumarins are toxic to many insects, reductions in the foliar concentration of these compounds may render plants in shady spots more vulnerable to herbivory. Although no specific phototoxin has been identified in wheat, shade reduces resistance of hard red spring wheat to the wheat stem sawfly Cephus cinctus; in this case, reduced photosynthetic efficiency may have reduced plant vigor or production by the plant of other defensive chemicals (51). Since many allelochemicals are induced by light, variation in light intensity can greatly affect the chemical composition of above ground plant parts (Berenbaum 1987).

Geographic variation may affect the efficacy of phototoxicity as a plant defense against insects. Global variation in the incidence of UV and visible light is substantial. Toxicity of a phototoxin can be directly proportional to UV intensity (Berenbaum and Zangerl 1987), so an equivalent concentration of phototoxin at a higher latitude, where incident UV is attenuated, may have reduced toxicity. Global radiation intensity is determined by a number of factors including solar angle, elevation above sea level, atmospheric ozone concentration, atmospheric turbidity, degree of cloud cover, and distance to the sun at any point in time (52). An increase in altitude from sea level to 4300 m corresponds to an increase in UV radiation of 66% (53). Latitudinal differences also affect UV intensities, largely due to global differences in the distribution of atmospheric ozone concentrations; greater concentrations of ozone at high latitudes greatly reduce the intensity of biologically effective UV radiation (Caldwell 1974). This sort of global variation in the distribution of UV radiation may account for the observation (54) that plant families with endogenous phototoxins appear to be more abundant in regions where intense solar radiation is available throughout most of the year (e.g., in tropical or arid desert ecosystems).

Conclusions

Many plant families have converged upon a common mechanism of defense against herbivorous insects, that is, to exploit the abundant energy available in sunlight to potentiate endogenous secondary chemicals. It is therefore hardly surprising that, over evolutionary time, herbivorous insects have developed various and sundry resistance mechanisms to these light-activated defense compounds. These include behavioral, physical and biochemical adaptations to reduce the extent of exposure to either the toxin or to potentiating wavelengths of light, or to dismantle and disarm the toxin itself. While light-activated phytochemicals may well have potential applications for control purposes in agricultural entomology, these phytochemicals may be as prone to counteradaptation by insects as are the more traditional synthetic organic control chemicals--perhaps more so, since there already exists a substantial group of insects preadapted to feeding on phototoxic plants. Moreover, there are ecological constraints on the use of phototoxins for widespread insect control. Local variations in light regime due to such uncontrollable factors as cloud cover or atmospheric turbidity, or to such unmodifiable factors as altitude or latitude, may render a standard phototoxin-based control program at best unpredictable.

Acknowledgments

Literature Cited

1. Berenbaum, M.; Neal, J.J. J. Chem. Ecol. 1986, 12, 809-812.
2. Fraenkel, G.S. Science 1959, 129, 1466-1470.
3. Berenbaum, M. Science 1978, 201, 532-534.
4. Croteau, R.; Burbott, A.J.; Loomis, W.D. Phytochem. 1972, 11, 2937-2948.
5. Arnason, T.; Swain, T.; Wat, C.K.; Graham, E.A.; Partington, S.; Towers, G.H.N. Biochem. System. Ecol. 1981, 9, 63-68.
6. McLachlan, D.; Arnason, J.T.; Philogene, B.J.R.; Champagne, D. Experientia 1982, 38, 1061-1062.
7. Champagne, D.E.; Arnason, J.T.; Philogene, B.J.R.; Morand, P.; Lam, J. J. Chem. Ecol., 1986, 12, 835-858.
8. Philogene, B.J.R.; Arnason, J.T.; Towers, G.H.N.; Campos, F.; Champagne, D.; McLachlan, D. J. Chem. Ecol. 1984, 10, 115-123.
9. Arnason, T.; Towers, G.H.N.; Philogene, B.J.R.; Lambert, J.D.H. Am. Chem. Soc. Symposium Ser. 1983, 208, 140-151.
10. Knox, J.P.; Dodge, A.D. Phytochem. 1985, 24, 889-896.
11. Kagan, J.; Szczepanski; Bindokas, V.; Wulff, W.; McCallum, J.S. J. Chem. Ecol. 1986, 12, 899-914.
12. Murray, R.H.; Mandez, J.; Brown, S. The Natural Coumarins; John Wiley and Sons, Ltd.: London.
13. Muckensturm, B.; Duplay, P.C.R.; Simonis, M.T.; Kienlen, J.C. Biochem. System. Ecol. 1981, 9, 289-292.
14. Yajima, T.; Kato, N.; Munakata, K. Agric. Biol. Chem. 1977, 41, 1263-1268.
15. Philogene, B.J.R.; Arnason, J.T.; Duval, F. Can. Ent. 1985, 117, 1153-1157.
16. Downum, K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest. Biochem. Physiol. 1984, 22, 104-109.
17. Kagan, J.; Chan, G. Experientia 1983, 39, 402-403.
18. Waller, G.R.; Nowacki, E.K. Alkaloid Biology and Metabolism in Plants; Plenum Press: New York, 1978.
19. Towers, G.H.N. Can. J. Bot. 1984, 62, 2900-11.
20. Pate, D.W. Econ. Bot. 1983, 37, 396-405.
21. Ohlsson, A.B.; Bjork, L.; Gatenbeck, S. Phytochem. 1983, 22, 2447-2450.
22. Arakawa, O.; Hori, Y.; Ogata, R. Physiologia Plantarum 1985, 3, 64.
23. Manuwoto, S.; Scriber, J.M. Ag. Ecosyst. Env., 1985, 14, 221-236.
24. Heller, W.; Egin-Buehler, B.; Gardiner, S.; Knobloch, K-H.; Matern, U.; Ebel, J.; Hahlbrock, K. Plant Physiol., 1979, 64, 371-373.
25. Hart, S.; Kogan, M.; Paxton, J. J. Chem. Ecol. 1983, 9, 657-672.
26. Schultz, J.C. In Variable Plants and Herbivores in Natural and Managed Systems; Denno, R.F. and McClure, M.S., Eds.; Academic Press: New York, 1983; pp. 61-90.
27. Lincoln, D.; Langenheim, J.H. Biochem. System. Ecol. 1978, 6, 21-32.
28. Ehrlich, P.; Raven, P. Evolution 1964, 18, 586-608.
29. Robberecht, R.; Caldwell, M.M. Plant, Cell, and Env. 1983, 6, 477-485.

30. Duran, N.; Song, P.-S.; Photochem. Photobiol., 1986, 43, 677–680.
31. Tietz, H.M. An Index to the Described Life Histories, Early Stages and Hosts of the Macrolepidoptera of the Continental United States and Canada; A.C. Allyn: Sarasota (FL), 1972.
32. Pathak, M.A.; Jimbow, K.; Szabo, G.; Fitzpatrick, T.B. Photochem. Photobiol. Rev. 1974, 1, 211–239.
33. Chapman, R.F. The Insects—Structure and Function; Elsevier: New York, 1971.
34. Gardiner, B.O.C. J. Res. Lep. 1976, 15, 184.
35. Mendez, J.; Brown, S.A. Can. J. Bot. 1971, 49, 2097–2100.
36. Safranek, L.; Riddiford, L.M. J. Insect Physiol. 1975, 21, 1931–1938.
37. Pathak, M.A.; Fitzpatrick, T.B. In Sunlight and Man; Fitzpatrick, T.B., Ed.; University of Tokyo Press: Tokyo, 1974; pp. 725–740.
38. Larson, R.A. J. Chem. Ecol. 1986, 12, 859–870.
39. Van Natto, C.; Freitag, R. Can. Entomol., 1986, 118, 89–96.
40. Czeczuga, B.; Biochem. System. Ecol., 1986, 14, 345–351.
41. Larson, R.A.; Marley, K.A. Phytochem. 1984, 23, 2351–2354.
42. Takahama, U.; Youngman, R.J.; Elstner, E.F. Photobiochem. Photobiophys. 1984, 7, 175–181.
43. Wilson, A. Phytochem. 1986, 25, 1309–1313.
44. Joshi, P.C.; Pathak, M.A. Biochem. Biophys. Res. Comm. 1983, 112, 638–646.
45. Rothschild, M; Mummery, R.; Farrell, C. Bio. J. Linn. Soc. 1986, 28, 359–372.
46. Feeny, P.; Rosenberry, L.; Carter, M. In Herbivorous Insects Host-seeking Behavior and Mechanisms; Ahmad, S., Ed.; Academic Press: New York, 1983; pp. 27–76.
47. Valadon, L.R.G.; Mummery, R.S. Comp. Biochem. Physiol. 1978, 61B, 359–372.
48. Bull, D.L.; Ivie, G.W.; Beier, R.C.; Pryor, N.W. J. Chem. Ecol., 1986, 12, 885–892.
49. Ivie, G.W.; Bull, D.L.; Beier, R.C.; Pryor, N.W. J. Chem. Ecol. 1986, 12, 871–884.
50. Berenbaum, M.; Zangerl., A.; Nitao, J. Phytochem., 1984, 23, 1809–1810.
51. Holmes, N.D. Can. Ent. 1984, 116, 677–684.
52. Caldwell, M.M. In Photophysiology; Giese, A.C., Ed.; 1971; Vol. 6, 131–177.
53. Caldwell, M.M. Ecol. Monog., 1968, 38, 243–268.
54. Downum, K.R.; Rodriguez, E.; J. Chem. Ecol., 1986, 12, 823–834.

Chapter 15

Biological Actions and Metabolic Transformations of Furanocoumarins

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Furanocoumarins are a group of potent photosensitizing molecules that occur as secondary constituents of a large number of plant families. Furanocoumarins have important uses in human medicine, are potent phototoxins to both man and domestic animals, are important host resistance mediators in a number of plant species, and exhibit toxicity against a wide range of organisms. Furanocoumarin biological actions are expressed most potently upon activation by long wavelength ultraviolet light, but these compounds also have light independent actions--by mechanisms that are at present not understood. In mammals, birds, and insects, furanocoumarins are often rapidly metabolized and excreted, and in insects, the rate of metabolism is the major determinant of relative tolerance to these compounds in the diet. Metabolic mechanism in animals include O-alkyl hydrolysis, hydrolysis of the pyrone ring, and oxidative opening of the furan ring, in addition to other oxidative, reductive, and conjugative reactions.

Furanocoumarins occur naturally as secondary metabolites in higher plants (1). These compounds have been isolated from well over a hundred plant species representing at least eight families, although the Umbelliferae and Rutaceae appear to have, in particular, large numbers of species that contain furanocoumarins (2).

Specific furanocoumarin derivatives generally arise in nature from two configurations of the basic tricyclic ring structure (Figure 1). The number of distinct furanocoumarin structures presently known from plants is quite large--well over 200 different furanocoumarin structures have thus far been identified (3). Most individual furanocoumarins are distinguished by alkoxy or alkyl substitution at either of the two available aromatic positions, at the available carbons of the furan ring or, much less predominantly,

Psoralen   Isopsoralen
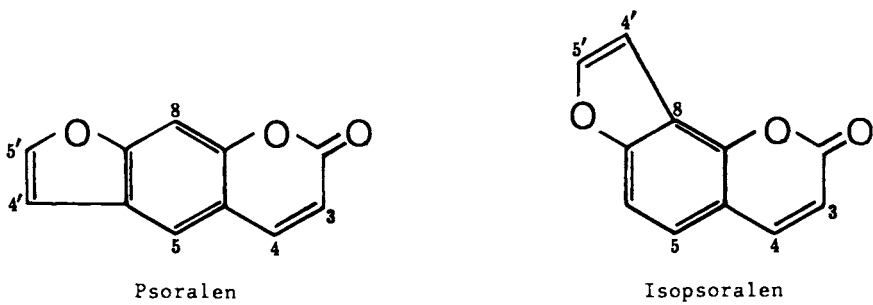
Figure 1. Structures indicating the ring fusion of linear
furanocoumarins (Psoralen) and angular furanocoumarins
(Isopsoralen).

the pyrone ring. Many naturally-occurring furanocoumarins exhibit
saturation of the furan olefinic moiety. Monosubstituted and
disubstituted furanocoumarins are common, but tri- or greater
substitution is rare. The pathways involved in the biosynthesis of
furanocoumarins have been thoroughly studied and are at present
quite well understood (4-6).

Mode of Biological Actions

Light Dependent Actions. Furanocoumarins are of interest from
agricultural, medicinal, public health, and environmental viewpoints
because they are highly photobiologically active. Wavelengths in
the near ultraviolet (320-360 nm) are the most effective activating
wavelengths, even though furanocoumarins absorb relatively poorly in
this region (7).
      It is generally accepted that the photobiological actions of
furanocoumarins result, in at least major part, from their
intercalation into the double helix of DNA where, upon light
activation, they form cyclobutane adducts with pyrimidine bases (8).
Both the furan and pyrone ring double bonds are potential alkylating
moieties; the linear furanocoumarins (psoralens) are known to form
both mono- and diadducts (crosslinks), whereas the angular
configuration of the isopsoralens permits only monoadduction (8).
      Although DNA photoalkylation is a well-defined molecular event
associated with furanocoumarin interactions with living matter,
recent studies have produced evidence that furanocoumarins bind with
specific, saturable, high affinity sites on or in mammalian cells
and that such binding is to some extent irreversible upon UV
exposure (9). It was proposed that specific receptor binding
phenomena as modes of action, rather than simply the alkylation of
DNA, might be more consistent with the known diversity of
furanocoumarin biological actions (9).

Light-Independent Actions. Although furanocoumarins are known
primarily for their light-catalyzed reactions, they nevertheless
have demonstrated biological activities in the absence of activating
radiation. Furanocoumarins are moderately toxic to laboratory
mammals in the dark, from both acute and subacute standpoints
(8,10). Furanocoumarins are also somewhat toxic to certain insects
in the dark, but such may result from antifeedant activity more so
than inherent toxicity per se (11). Some furanocoumarins are weakly
mutagenic in the absence of light, by thus far unexplained
mechanisms (12-16). Certain of the environmental effects of
furanocoumarins (vide infra) are clearly light independent but,
again, the mechanisms involved are not known.

      Role of Oxygen in Furanocoumarin Action. In the photoadduction
of furanocoumarins with DNA, there is clearly no direct involvement
of oxygen. However, the photogeneration of singlet oxygen by
furanocoumarins has been well documented, and such reactions may
potentially be responsible for direct enzyme inactivation and
membrane disruption (17). Monofunctional (angular) furanocoumarins
appear to be more efficient generators of singlet oxygen than are
linear furanocoumarins (17). They also appear to be more
photocarcinogenic than the bifunctional (linear) furanocoumarins,

and it has been suggested that activated oxygen may play a major role in the photocarcinogenicity of these compounds (2). Other studies, however, have shown that singlet oxygen generation is not significantly correlated with the genotoxicity of either linear or angular furanocoumarins, using an excision repair deficient strain of E. coli as the test organism (18). Similarly, neither singlet oxygen (19) nor superoxide radical (20) formation could be correlated with skin photosensitizing activity amongst a considerable array of linear and angular furanocoumarins.

Structure-Activity Considerations

Available data on the skin photosensitizing activity of furanocoumarins indicate that, generally, linear furanocoumarins are more biologically active than the angular analogs (8). Recent studies have, however, provided data to suggest that under natural conditions of multiwavelength light activation, the inherent biological activities of comparable linear and angular furanocoumarins may not be appreciably different (21). It is also generally held that photosensitizing activity decreases with increasing chemical complexity of the alkyl or alkoxy substituent (8), but some studies have shown that some of the more chemically complex (and more polar) furanocoumarins are highly phototoxic when the skin, a barrier to absorption, is bypassed (22). Aryl hydroxylated furanocoumarins, several of which occur in nature, are inactive as skin photosensitizers (8). Structure-activity correlations for furanocoumarins with respect to biological actions other than skin photosensitization are either incomplete or lacking.

Toxicological and Other Biochemical Effects

Because furanocoumarins are potent DNA photoalkylating agents, it is not surprising that they show considerable phototoxicity toward a wide variety of life forms. Upon light activation, furanocoumarins are powerful antimicrobial agents (8,23), nematocides (24), insecticides (25,26), ovicides (27), and powerful skin photosensitizers against man (8) and animals (28). They are also possibly herbicidal (29). Furanocoumarins are molluscicidal (30,31) and piscicidal (8), but the role of light in these effects is unclear. Considering the known molecular events associated with the light-sensitized interaction of furanocoumarins with living matter, it seems almost certain that furanocoumarins in the presence of activating light would be potentially phototoxic to almost any form of life.

In addition to acute phototoxicological effects, furanocoumarins are highly photomutagenic and are, in fact, mammalian photocarcinogens (32-34).

Furanocoumarins are known to have various light independent effects on some mammalian and insect enzyme systems. The linear furanocoumarin, xanthotoxin (8-methoxypsoralen) induced mouse and/or rat aryl hydrocarbon hydroxlyase (35,36), ethyl morphone N-demethylase (36), p-nitroanisole-O-demethylase (35) and 7-ethoxycoumarin-O-deethylase (37); it also shortened hexobarbital sleeping time (38), and it may increase levels of cytochrome P-450

(36) although there are conflicting data (37). Xanthotoxin did not
induce aniline hydroxylase (36). Interestingly, psoralen,
isopsoralen, and 4,5',8-trimethylpsoralen at equivalent doses failed
to exhibit any enzyme induction potential (35,36). In the fall
armyworm (Spodoptera frugiperda) dietary xanthotoxin induced midgut
glutathione S-transferase and heptachlor epoxidase, increased
cytochrome P-450 content, but inhibited aldrin epoxidase,
biphenyl-4-hydroxylase, and p-chloro-N-methyl aniline N-demethylase
(39).

Environmental Interactions

On the basis of research observations to date, the most plausible
explanation for the occurrence of furanocoumarins in higher plants
is that these compounds evolved as defense chemicals against plant
pathogens and herbivores, and as allelopathic agents to enhance
competitiveness amongst other plant species. Furanocoumarins
inhibit seed germination (40) and plant growth (41)--activities that
are almost certainly light-independent because of their expression
in the soil environment. The avoidance of autotoxicity is
apparently accomplished, at least in part, through localization
and/or sequestration phenomena (40).
     Furanocoumarins are well established as phytoalexins. The
infection of both celery and parsnip with certain pathogenic
organisms results in greatly enhanced biosynthesis and accumulation
of these compounds (42); enhanced biosynthesis of furanocoumarins is
also elicited by a number of other stimuli as well (43).
     The antifeedant properties of furanocoumarins are well
established for a number of insects, including several Spodoptera
species (25,44-46), Mythimna unipunctata (47), and Leptinotarsa
decemlineata (47). Conversely, insects that are adapted to feed on
furanocoumarin-containing plants may perceive these compounds as
oviposition stimulants (48), and in at least one insect, the black
swallowtail butterfly, (Papilio polyxenes), dietary furanocoumarins
actually enhance caterpillar growth rate and weight gain, perhaps by
acting as feeding stimulants (49).
     Furanocoumarins in plants pose clear and documented hazards to
grazing mammals. Photosensitization of domestic cattle, sheep, and
poultry by dietary furanocoumarins has been documented (28), and
there are numerous instances of photosensitization in man associated
with dermal plant exposures (8). The impact of furanocoumarins on
mammalian and avian wildlife species is essentially unknown, but it
is likely that most wildlife species have adapted through
evolutionary pressures to avoid such plants. Evidence has recently
been obtained that furanocoumarins are probably antifeedants toward
at least one mammalian herbivore, the hyrax (Procavia capensis)
(50).

Medicinal Uses

The various photobiological actions exhibited by furanocoumarins are
such that these compounds have an astounding range of actual and
potential uses in human medicine. Plant preparations that contain
furanocoumarins, plus sunlight, have been used for thousands of
years in the treatment of vitiligo (8), and xanthotoxin plus UV

light (PUVA therapy) is now the treatment of choice for severe
psoriasis (32,33). Other human disorders for which furanocoumarins
(usually xanthotoxin) plus light have shown potentially beneficial
effects include mycosis fungoides (51), scleromyxoedma (52),
urticaria pigmentosa (53,54), follicular mucinosis (55),
parapsoriasis (56), palmoplantar pustulosis (57), lymphomatoid
papulosis (58), lichen planus (59), atopic dermatitis (60), certain
parasitic fungi (61), and even herpes simplex (62), alopecia areata
(63,64), and leukemic cutaneous T cell lymphoma (65).

Metabolic Transformations

Mammals. The extensive interest in linear furanocoumarins as
medicinal agents and their impact as livestock and poultry
phototoxins has led to considerable scientific efforts aimed at
elucidating the kinetics, biotransformation and disposition of these
chemicals in mammalian species, including man. Most such efforts
have been targeted on xanthotoxin, because it is the furanocoumarin
of choice in most medicinal applications, is perhaps the most
commonly-occurring furanocoumarin in nature, and is highly
photobiologically active. However, limited biological
transformation studies have also been done with bergapten
(5-methoxypsoralen), and with 4,5',8-trimethylpsoralen which is
clinically useful in the treatment of vitiligo.
    The in vivo and in vitro metabolism of xanthotoxin in
laboratory rats and mice has been the subject of several studies
(66-70). In rodents, xanthotoxin is metabolized by 1)
O-demethylation; 2) aryl hydroxylation at position 5; 3) oxidation
of the 5,8-dihydroquinone to the quinone; 4) hydrolysis of the
pyrone ring; 5) oxidative opening of the furan ring; and 6) sulfate
and glucuronide conjugation (Figure 2). In vitro studies have
demonstrated that xanthotoxin metabolism in rats is induced by
phenobarbital and by B-naphthoflavone (70). In rats, there is some
indication that cleavage of the aromatic ring of xanthotoxin occurs
to a very limited extent (69).
    As indicated in Figure 2, xanthotoxin metabolism in the dog
(71), in the goat (72), and in man (67,73-75) follows at least some
of the same pathways. In the goat, a novel metabolite results from
saturation of the pyrone ring prior or subsequent to pyrone ring
hydrolysis. Although not established experimentally, this
metabolite may arise through reductive mechanisms present in the
rumen prior to absorption (72).
    Definitive metabolic fate studies have not been undertaken with
bergapten, but a single study of limited scope has provided good
evidence that, in man, the pyrone ring of bergapten is hydrolyzed
and subsequently conjugated with glucuronic acid or sulfate prior to
excretion in the urine (76).
    Studies with 4,5',8-trimethylpsoralen in mouse and man have
shown that the 5'-methyl group is hydroxylated, then oxidized to a
5'-carboxy derivative (Figure 3) (77,78). Products of furan or
pyrone ring cleavage reactions were not detected.

Birds. There are no published data on the fate of furanocoumarins
in any avian species, but studies in progress in our laboratories
have shown that xanthotoxin is extensively metabolized by laying
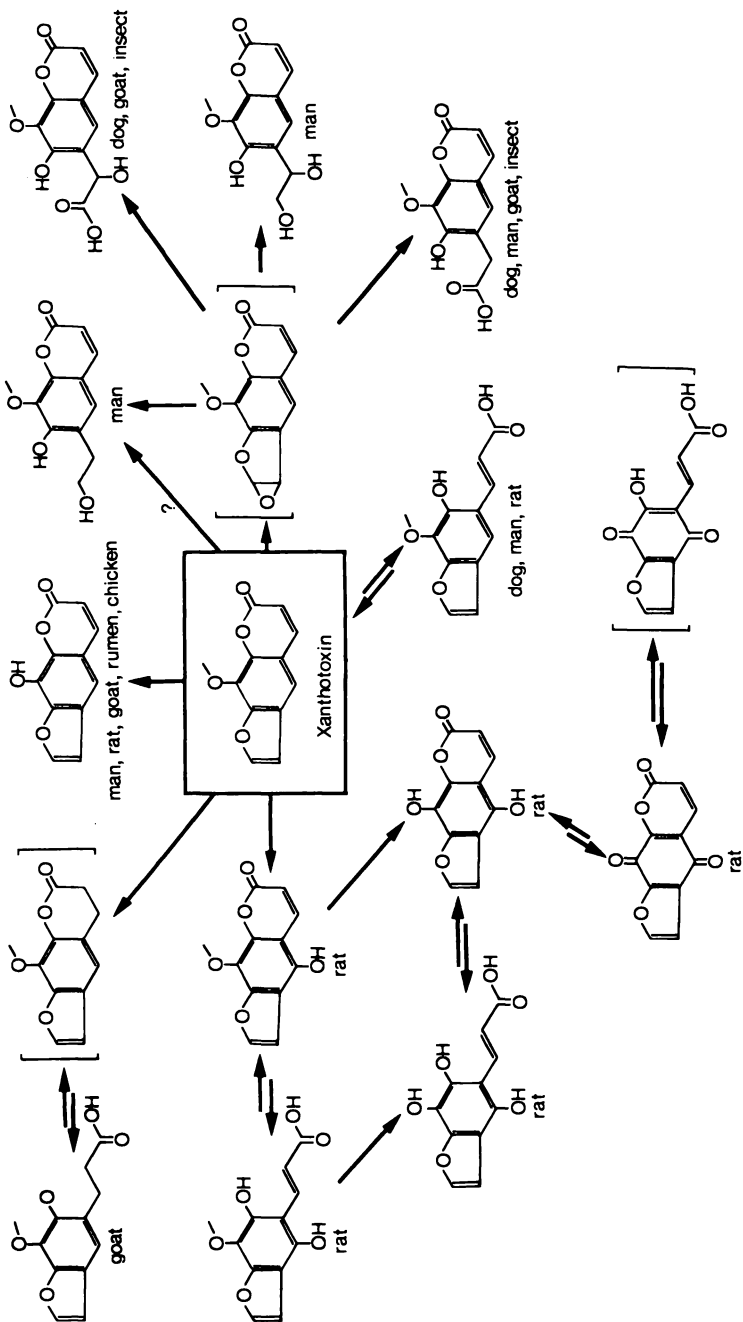
Figure 2. Identified metabolic pathways for xanthotoxin (8-methoxypsoralen) in rodents, humans, dogs, goats, chickens, and insects. Bracketed compounds have not been isolated, but are possible intermediates in pathways leading to the identified metabolites.
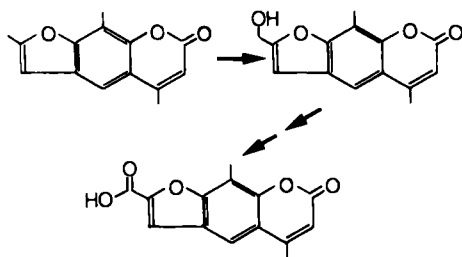
Figure 3.   Metabolites of 4,5',8-trimethylpsoralen in mice and humans.

hens (Pangilinan, N. C.; Ivie, G. W.; unpublished data). The hydrolysis of the O-methyl group of xanthotoxin (to xanthotoxol) occurs to at least a limited extent in the hen, and preliminary indications are that the major metabolites arise through furan and/or pyrone ring cleavage reactions.

Insects. A number of studies have investigated the disposition of furanocoumarins in insects, with the primary emphasis on establishing how furanocoumarin-tolerant species avoid phototoxicity. Under conditions of laboratory feeding of xanthotoxin to aphids (Aphis heraclella or Cavariella pastinacae) continuously exposed to UV light, no phototoxic effect was seen. No xanthotoxin metabolites per se were detected in extracts of treated aphids, but xanthotoxin could be liberated by acid hydrolysis procedures (29). It may be that aphids detoxify xanthotoxin by hydrolysis of the lactone followed by conjugation (perhaps as a glycoside). Acid hydrolysis could yield the free hydroxy acid which then would likely lactonize back to xanthotoxin.

The larval form of a leaf mining dipteran, Phytomyza spondylii, feeds on plants rich in furanocoumarins and has been shown to rapidly detoxify xanthotoxin to non-photoactive metabolites, although the chemical nature of these products has not been investigated (79).

Caterpillars of the black swallowtail butterfly, Papilio polyxenes, are well adapted to feed on linear furanocoumarin rich host plants, and it is now known that this insect rapidly detoxifies linear furanocoumarins (psoralen, xanthotoxin) as the mechanism of toxicity avoidance (80,81). Oxidative cleavage of the furan ring is the major route of detoxification by P. polyxenes (Figure 2); O-demethylation, pyrone ring hydrolysis, or other pathways are either non-existent or minor. Larvae of the furanocoumarin-sensitive fall armyworm (Spodoptera frugiperda) metabolize linear furanocoumarins by identical pathways, yet at such a slow rate that toxicity ensues (80,81).

P. polyxenes appears to be relatively less tolerant to angular furanocoumarins (82) and, indeed, an angular furanocoumarin (isopsoralen) was metabolized at a slower rate than was psoralen (83). This observation may at least partly explain why P. polyxenes generally avoids plants that contain appreciable levels of the angular compounds.

The metabolic detoxification of furanocoumarins in lepidopteran larvae results, at least in part, from the actions of microsomal oxidases. This conclusion is based on direct studies with xanthotoxin and midgut or body microsomes from P. polyxenes and S. frugiperda (84), and upon observations that the toxicity (in the dark) of xanthotoxin to the corn earworm, Heliothis zea, is enhanced by myristicin and piperonyl butoxide, potent methylenedioxyphenyl inhibitors of microsomal oxidase enzymes (11).

Metabolism Versus Expression of Biological Effects

Given the fact that furanocoumarins readily photoalkylate DNA, these compounds can be considered as nonspecific photosensitizers capable of interacting with essentially any form of life under appropriate light activation scenarios. In single celled organisms, such

interactions may lead to cellular death, the inhibition of cellular division and thus multiplication or, at a minimum, mutagenic effects. In multicellular organisms, the photosensitized effects are expected to be limited to dermal and subdermal tissues, i.e., limited by the degree of light penetration. In mammals, particularly, such interactions may be toxicological in that severe tissue damage occurs (erythema, skin blistering, carcinogenicity) or pharmacological in that furanocoumarin-light-tissue interactions result in desired medicinal effects (vitiligo, psoriasis).

The rate of furanocoumarin metabolism by any organism almost certainly governs the severity and duration of the photobiological actions associated with these compounds. This conclusion seems justified in that essentially any likely metabolic transformation can be expected to result in significant or total diminution of photoreactivity and/or an increased tendency toward rapid excretion. Aryl hydroxylation or O-alkyl hydrolysis reactions render furanocoumarins photobiologically inactive (8) and, on structural grounds, furan or pyrone ring cleavage should result in inactive (although not yet established experimentally) and easily excreted metabolites. Certain of the potential intermediates in furanocoumarin metabolism might, in fact, retain photobiological activity (i.e., the 4',5'-epoxide, the quinone, and the 3,4-dihydro derivatives, Figure 2). However, such compounds would be rapidly subjected to additional degradation reactions. With 4,5',8-trimethylpsoralen, mammalian metabolism apparently involves methyl group oxidation to a greater extent than ring cleavage (if indeed ring cleavage reactions occur at all), but the major metabolite (5'-carboxy dimethylpsoralen) is photobiologically inactive (77) and readily excreted. Reduced biological activity of furanocoumarin metabolites has also been indicated by an observed reduction in photomutagenic activity of xanthotoxin after incubation with rat liver mixed function oxidase enzymes (85).

The metabolism of furanocoumarins by higher organisms appears to be, almost universally, quite rapid. In orally-dosed rodents and in man, peak plasma levels of absorbed xanthotoxin usually occur within 1-2 hours, followed by rapid depletion (67,74,75). Metabolites are quickly, and primarily, eliminated in the urine (68,74,75). Similarly, rapid rates of metabolic detoxification and excretion are seen in dogs (71), the goat (72), and in birds (Pangilinan, N. C.; Ivie, G. W.; unpublished data). Even in insects not adapted to dietary furanocoumarins (S. frugiperda), metabolism and excretion are quite rapid, although far less so than for the furanocoumarin tolerant P. polyxenes. Six hours after oral treatment of S. frugiperda with xanthotoxin, only about 6% of the administered dose remains unexcreted as the parent compound (81).

Although furanocoumarins are in general much more biologically active in the presence of long wavelength UV light, these compounds do have demonstrable light-independent actions (vide supra). It is, in general, poorly known to what extent biotransformations might affect such actions, although it is probably true that ultimate metabolism would usually result in derivatives of decreased biological activity (see Figure 2). However, rat liver enzymes, in vitro, apparently metabolize both xanthotoxin and 4,5',8-trimethyl= psoralen to mutagenic derivatives (14). Also, some synthetic angular furanocoumarins (with hydrophilic substituents at the 4'

position of the furan ring) are mutagenic in the dark, but only
after microsomal activation (87). The nature of the mutagenic
metabolites of such compounds is unknown. That methylenedioxyphenyl
compounds synergize the toxicity of xanthotoxin to <u>Heliothis</u> in the
dark (11) implies that the actions of mixed function oxidase enzymes
in this insect are primarily of a detoxification nature.
    The effects of metabolic alterations of potentially
biologically active substituent moieties of furanocoumarins is also
poorly understood. It is known that the synthetic furanocoumarin
derivative, psoralen glycidyl ether, is a potent light independent
mutagen, but that the action of epoxide hydrolases reduces
mutagenicity, clearly through hydrolysis of the epoxide moiety (86).

Conclusions

Furanocoumarins have perhaps the widest documented spectrum of
biological activities of any class of organic chemicals yet studied.
Because of their major light-catalyzed mode of interaction with
living matter, presently well defined, furanocoumarins can
potentially exert photobiochemical influences on essentially any
life form. Furanocoumarins also have significant light-independent
actions, by mechanisms that are, at present, essentially unstudied.
    Studies of the biochemical fate of furanocoumarins in a number
of vertebrate and invertebrate species have provided data of
considerable value in establishing how these chemicals interact with
various life forms and in explaining the relative photosensitivity
of different species to furanocoumarins. Additional mechanistic and
fate studies are, however, clearly needed to assess the potential
role of non DNA-alkylation modes-of-action (i.e., receptor binding)
on both the light-dependent and light-independent actions of these
toxicologically, pharmacologically, agriculturally, and
environmentally significant compounds.

Literature Cited

1. Perone, V. B. <u>Microbial Toxins</u> 1972, 8, 67-92.
2. Towers, G. H. N. <u>Can. J. Bot.</u> 1984, 62, 2900-2911.
3. Murray, R. D. H.; Mendez, J.; Brown, S. A. <u>The Natural</u>
   <u>Coumarins</u>; Wiley: New York, 1982, 702 pp.
4. Caporale, G.; Innocenti, G.; Guiotto, A.; Rodighiero, P.;
   Dall'Acqua, F. <u>Phytochemistry</u>, 1981, 20, 1283-1287.
5. Innocenti, G.; Dall'Acqua, F.; Caporale, G. <u>Phytochemistry</u>
   1983, 22, 2207-2209.
6. Rodighiero, P.; Guitto, A.; Pastorini, G.; Manzini, P.;
   Dall'Acqua, F.; Innocenti, G.; Caporale, G. <u>Gazz. Chim. Ital.</u>
   1980, 110, 167-172.
7. Towers, G. H. N. <u>Prog. Phytochem.</u> 1980, 6, 183-202.
8. Scott, B. R.; Pathak, M. A.; Mohn, G. R. <u>Mutat. Res.</u> 1976, 39,
   29-74.
9. Laskin, J. D.; Lee, E.; Yurkow, E. J.; Laskin, D. L.; Gallo,
   M. A. <u>Proc. Natl. Adad. Sci.</u> 1985, 82, 6158-6162.
10. Emerole, G.; Thabrew, M. I.; Anosa, V.; Okorie, D. A.
    <u>Toxicology</u> 1981, 20, 71-80.

11. Berenbaum, M.; Neal, J. J. J. Chem. Ecol. 1985, 11, 1349-1358.
12. Bridges, B. A.; Mottershead, R. P. Mutat. Res. 1977, 44,
     305-312.
13. Wottawa, A.; Viernstein, H. Mutat. Res. 1981, 85, 298-299.
14. Kirkland, D. J.; Creed, K. L.; Mannisto, P. Mutat. Res. 1983,
     116, 73-82.
15. Quinto, I.; Averbeck, D.; Moustacchi, E.; Hrisoho, Z.; Moron,
     J. Mutat. Res. 1984, 136, 49-54.
16. Uwaifo, A. O. Life Sci. Adv. 1984, 3, 62-70.
17. Knox, J. P.; Dodge, A. D. Phytochemistry 1985, 24, 889-896.
18. Tamaro, M.; Babudri, N.; Pani, B.; Baccichetti, F.; Rodighiero,
     P. Med. Biol. Environ. 1983, 11, 493-497.
19. Vedaldi, D.; Dall'Acqua, F.; Rodighiero, G. Med. Biol.
     Environ. 1983, 11, 507-508.
20. Vedaldi, D., Dall'Acqua, F.; Bollettin, P.; Rodighiero, G.
     Med. Biol. Environ. 1984, 12, 569-573.
21. Potapenko, A. Y.; Sukhorukov, V. L.; Davidov, B. V.
     Experientia 1984, 40, 264-265.
22. Ivie, G. W. J. Agric. Food Chem. 1978, 26, 1394-1403.
23. Fowlks, W. L.; Griffith, D. G.; Oginsky, E. L. Nature 1958,
     181, 571-572.
24. Fujita, H.; Ishii, N.; Suzuki, K. Photochem. Photobiol. 1984,
     39, 831-834.
25. Berenbaum, M. Science 1978, 201, 532-534.
26. Philogene, B. J. R.; Arnason, J. T.; Duval, F. Can. Entomol.
     1985, 117, 1153-1157.
27. Kagan, J.; Chan, G. Experientia 1983, 39, 402-403.
28. Ivie, G. W. In Effects of Poisonous Plants on Livestock;
     Keeler, K.; Van Kampen, K.; James, L. Ed.; Academic: New York,
     1978, p 475.
29. Camm, E. L., Wat, C.-K.; Towers, G. H. N. Can. J. Bot. 1976,
     54, 2562-2566.
30. Schonberg, A.; Latif, N. J. Am. Chem. Soc. 1954, 76, 6208.
31. Marston, A.; Hostettmann, K. Phytochemistry 1985, 24,
     639-652.
32. Reshad, H.; Challoner, F.; Pollock, D. J.; Baker, H. Brit. J.
     Dermatol. 1984, 110, 299-305.
33. Stern, R. S.; Laird, N.; Melski, J.; Parrish, J. A.;
     Fitzpatrick, T. B.; Bleich, H. L. N. Engl. J. Med. 1984, 310,
     1156-1161.
34. Farber, E. M.; Abel, E. A.; Cox, A. J. Arch. Dermatol. 1983,
     119, 426-431.
35. Mandula, B. B.; Pathak, M. A.; Nakayama, Y.; Davidson, S. J.
     Br. J. Dermatol. 1978, 99, 687-692.
36. Bickers, D. R.; Mukhtar, H.; Molica, Jr., S. J.; Pathak, M. A.
     J. Invest. Dermatol. 1982, 79, 201-205.
37. Tsambaos, D.; Vizethum, W., Goerz, G. Arch. Dermatol. Res.
     1978, 263, 339-342.
38. Woo, W. S.; Lee, C. K.; Shin, K. H. Planta Med. 1982, 45,
     234-236.
39. Yu, S. J. Pestic. Biochem. Physiol. 1984, 22, 60-68.
40. Friedman, J.; Rushkin, E.; Waller, G. R. J. Chem. Ecol. 1982,
     8, 55-65.

41. Shimomura, H.; Sashida, Y.; Nakata, H.; Kawasaki, J.; Ito, Y. Phytochemistry 1982, 21, 2213-2215.
42. Ivie, G. W.; Holt, D. L.; Ivey, M. C. Science 1981, 213, 909-910.
43. Beier, R. C.; Ivie, G. W.; Oertli, E. H. In Xenobiotics in Foods and Feeds; Finley, J. W.; Schwass, D. E., Ed.; ACS Symp. Ser. 234, 1983; Chapter 19, p 295.
44. Gebreyesus, T.; Chapya, A. Curr. Themes Trop. Sci. 1983, 2, 237-242.
45. Yajima, T.; Kato, N.; Munakata, K. Agric. Biol. Chem. 1977, 41, 1263-1268.
46. Yajima, T.; Munakata, K. Agric. Biol. Chem. 1979, 43, 1701-1706.
47. Muckensturm, B.; Duplay, D.; Robert, P. C.; Simonis, M. T.; Kienlen, J. C. Biochem. Syst. Ecol. 1981, 9, 289-292.
48. Stadler, E.; Buser, H.-R. Experientia 1984, 40, 1157-1159.
49. Berenbaum, M. Ecol. Entomol. 1981, 6, 345-351.
50. Ashkenazy, D.; Kashman, Y.; Nyska, A.; Friedman, J. J. Chem. Ecol. 1985, 11, 231-239.
51. Adams, R.; Boyle, J.; Lever, R.; McQuillan, I.; MacKie, R. Scott. Med. J. 1982; 27, 264.
52. Farr, P. M.; Ive, F. A. Br. J. Dermatol. 1984, 110, 347-350.
53. James, M. P. Clin. Exp. Dermatol. 1982, 7, 311-320.
54. Vella Briffa, D.; Eady, R. A. J.; James, M. P.; Gatti, S.; Bleehen, S. S. Br. J. Dermatol. 1983, 109, 67-75.
55. Kenicer, K. J. A.; Lakshmipathi, T. Br. J. Dermatol. (Suppl.) 1982, 107, 48-49.
56. Powell, F. C.; Spiegel, G. T.; Muller, S. A. Mayo Clin. Proc. 1984, 59, 538-546.
57. Paul, R.; Jansen, C. T. Dermatologica 1983, 167, 283-285.
58. Wantzin, G. L.; Thomsen, K. Br. J. Dermatol. 1982, 107, 687-690.
59. Ortonne, J. P.; Thivolet, J.; Sannwald, C. Br. J. Dermatol. 1978, 99, 77-88.
60. Anderson, T. F.; Voorhees, J. J. Annu. Rev. Pharmacol. Toxicol. 1980, 20, 235-257.
61. Knudsen, E. A. Acta Derm. Venereol. 1980, 60, 452-456.
62. Thiers, B. H. J. Am. Acad. Dermatol. 1982, 7, 811-816.
63. Claudy, A. L.; Gagnaire, D. Acta Derm. Venereol. 1980, 60, 171-172.
64. Claudy, A. L.; Gagnaire, D. Arch. Dermatol. 1983, 119, 975-978.
65. Edelson, R.; Berger, C.; Gasparro, F.; Lee, K.; Taylor, J. Clin. Res. 1983, 31, 467A.
66. Nozu, T.; Suwa, T.; Migita, Y.; Tanaka, I. So Oyo Yakuri 1979, 18, 497-505.
67. Smyth, R. D.; Van Harken, D. R.; Pfeffer, M.; Nardella, P. A.; Vasiljev, M.; Pinto, J. S.; Huttendorf, G. H. Arzneim.-Forsch./Drug Res. 1980, 30, 1725-1730.
68. Mays, D. C.; Rogers, S. L.; Guiler, R. C.; Sharp, D. E.; Hecht, S. G.; Staubus, A. E.; Gerber, N. J. Pharmacol. Exp. Ther. 1986, 236, 364-373.
69. Wamer, W.; Giles, A.; Brouwer, E.; Kornhauser, A. Clin. Res. 1980, 28, 584A.

70. Sharp, D. E.; Mays, D. C.; Rogers, S. L.; Guiler, R. C.; Hecht, S.; Gerber, N. Proc. West. Pharmacol. Soc. 1984, 27, 255-258.
71. Kolis, S.; Williams, T.; Postma, E.; Sasso, G.; Confalone, P.; Schwartz, M. Drug Metab. Dispos. 1979, 7, 220-225.
72. Ivie, G. W.; Beier, R. C.; Bull, D. L.; Oertli, E. H. Am. J. Vet. Res. 1986, 47, 799-803.
73. Ehrsson, H.; Eksborg, S.; Wallin, I. Eur. J. Drug Metab. Pharmacokinet. 1978, 2, 125-128.
74. Busch, U.; Schmid, J.; Koss, F. W.; Zipp, H.; Zimmer, A. Arch. Dermatol. Res. 1978, 262, 255-265.
75. Schmid, J.; Prox, A.; Reuter, A.; Zipp, H.; Koss, F. W. Eur. J. Drug Metab. Pharmacokinet. 1980, 5, 81-92.
76. Stolk, L. M. L.; Westerhof, W.; Cormane, R. H.; Van Zwieten, P. A. Br. J. Dermatol. 1981, 105, 415-420.
77. Mandula, B. B.; Pathak, M. A.; Dudek, G. Science 1976, 193, 1131-1134.
78. Mandula, B. B.; Pathak, M. A. Biochem. Pharmacol. 1979, 28, 127-132.
79. Ashwood-Smith, M. J.; Ring, R. A.; Liu, M.; Phillips, S.; Wilson, M. Can. J. Zool. 1984, 62, 1971-1976.
80. Ivie, G. W.; Bull, D. L.; Beier, R. C.; Pryor, N. W.; Oertli, E. H. Science 1983, 221, 374-376.
81. Bull, D. L.; Ivie, G. W.; Beier, R. C.; Pryor, N. W.; Oertli, E. H. J. Chem. Ecol. 1984, 10, 893-911.
82. Berenbaum, M.; Feeny, P. Science 1981, 212, 927-929.
83. Ivie, G. W.; Bull, D. L.; Beier, R. C.; Pryor, N. W. J. Chem. Ecol. 1986, 12, 871-884.
84. Bull, D. L.; Ivie, G. W.; Beier, R. C.; Pryor, N. W. J. Chem. Ecol. 1986, 12, 885-892.
85. Schimmer, O.; Fischer, K. Mutat. Res. 1980, 79, 327-330.
86. Ivie, G. W.; MacGregor, J. T.; Hammock, B. D. Mutat. Res. 1980, 79, 73-77.
87. Monti-Bragadin, C.; Tamaro, M.; Venturini, S.; Pani, B.; Babudri, N.; Baccichetti, F. Farmaco Ed. Sci. 1981, 36, 551-556.

Chapter 16

Fungicidal Activity of Naturally Occurring Photosensitizers

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In recent years a diversity of natural photosensitizers with in vitro fungicidal activity has been described. Many type I photosensitizers photobind to DNA, including linear and angular furanocoumarins, furanochromones, furanoquinolines, and probably the β-carboline alkaloids. Isoflavonoids and aliphatic polyacetylenes attack membrane targets via a free radical mechanism. Aromatic polyacetylenes display competing type I and type II reactions and the thiophenes are strictly photodynamic sensitizers. The evidence for these toxic mechanisms is discussed, and evidence for the involvement of these phytochemicals resistance to fungal attack in vivo is reviewed.

Daniels (1) has described a simple and economic procedure which permits the rapid screening of plants and plant extracts for photo-toxic activity. This technique was originally used to identify fura-nocoumarins as the compounds responsible for the photosensitizing action of various umbelliferous plants. More recently, an ever increasing number of phototoxic secondary metabolites, including alkaloids, phenolics, quinones, terpenoids, and acetylenes and their thiophene derivatives have been isolated from vascular plants, fungi and bacteria. As yeasts, (particularly Candida, Saccharomyces, and Rhodotorula), and other fungi, are used in these assays, most of the known photosensitizers are fungicidal, although it is not always clear that such activity reflects the role of these compounds in the plant.

Photosensitizers vary in both their mechanisms of action and target sites within the cell. The two mechanisms recognized, termed type I and type II, are reviewed by C.S. Foote elsewhere in this volume and will be described only briefly here. In both type I and type II reactions the ground-state sensitizer is photoexcited to the unstable singlet state, followed by intersystem crossing to yield the longer-lived triplet sensitizer (2,3). In type I reactions the triplet sensitizer participates in radical or electron transfer reactions with susceptible biomolecules, thus consuming the sensi-tizer. Type II sensitizations involve the transfer of excitation

energy from the triplet sensitizer to ground-state (triplet) molecular oxygen, returning the sensitizer to its ground state and generating singlet oxygen. The sensitizer may subsequently be reexcited and catalyze further reactions. As type II sensitizations require the participation of molecular oxygen, the activity of such photosensitizers is abolished under anaerobic conditions and can be modified by azide or D_2O, which alter the lifetime of singlet oxygen. Some photosensitizers display an intermediate mechanism in which both type I and type II reactions occur competitively.

Potentially phototoxic secondary metabolites are known to occur in over thirty families of vascular plants; in some families a single species may elaborate several classes of photosensitizers derived from independant biosynthetic routes (3). The function of these compounds is not easy to establish, but their broad-spectrum biocidal activity against not only fungi but also bacteria and insects, and their frequent involvement in phytoalexin responses, strongly suggests that they function as part of a generalized defense against pathogens and herbivores. Of course, this does not preclude the possibility of simultaneous functions involving primary metabolism as well.

Type I Photosensitizers

Type I photosensitizers may covalently bind to a variety of suscep-tible target molecules, including proteins and tRNA (5-8), but the majority appear to form adducts with DNA, and so may be termed photogenotoxic (9-11). Such compounds are typically planar, tricyclic molecules.

The best known and first described of the photogenotoxins are the furanocoumarins, characteristic secondary metabolites of the Rutaceae, Apiaceae (Umbelliferae), and certain other families of flowering plants. Daniels (1) first showed that furanocoumarins cause lethal damage to yeasts in light, and this was subsequently confirmed in numerous studies with yeasts and other fungi (12-20). Toxicity results mainly from photobinding to the pyrimidine bases of DNA by means of double bonds at the 3,4 and 4',5' sites, forming monoadducts (21,22) or, in the case of some linear psoralens, bifunctional adducts leading to interstrand cross-linkages (23-27). This photoactivity is clearly ecologically relevant, as furanocoumarins are involved in the phytoalexin response to fungal infection in some umbellifers (28-30) and these compounds can photosensitize insects and other herbivores (31).

Other compounds which display this type of activity are the furanochromones (32), furanochromenes (33), furanoquinolines, and certain tryptophan-derived alkaloids including the β-carbolines (34-36). The best understood of these are the furanoquinoline alkaloids, particularly dictamnine (I), which occur in a number of rutaceous species including Skimmia japonica and Dictamnus albus (37). Dictamnine, skimmianine (II), maculoside, and maculine were phototoxic to the yeasts Saccharomyces cerevisiae and Candida albicans in UVA (36); dictamnine was also phototoxic to filamentous phytoparasitic and zooparasitic fungi including Mucor hiemalis, M. rammanianus, Fusarium graminearus, and Penicillium italicum (38). Both dictamnine and skimmianine inhibited mitosis and caused gross

chromosomal changes to Chinese hamster ovary (CHO) cells in UVA but net in the dark, suggesting a cellular target in the nucleus, similar to the furanocoumarins (39). Subsequently it was shown that [^{3}H]-dictamnine intercalates with calf-thymus DNA in the dark, and after near-UV irradiation and gel filtration to separate the unbound alkaloid, the label was found to be bound to the DNA (40). Hydroxyapatite chromatography of heat-denatured [^{3}H]-dictamnine-DNA complex showed only single-stranded DNA, indicating the formation of monoadducts. Studies of the photobinding of dictamnine towards various synthetic DNA's showed that the ratio of binding to poly(dA-dT)·poly(dA-dT):poly(dG-dC)·poly(dG-dC):poly(dA-dU)·poly (dA-U):-poly(dA)·poly(dT), in relation to that of calf thymus DNA, was 18:1:0.5:0.3, similar to the ratio observed for the furanocoumarin 8-methoxypsoralen (8-MOP). In addition, prior treatment of DNA with dictamnine greatly reduced incorporation of 8-MOP, suggesting that the binding sites for the two compounds are probably identical. Template activity of the photomodified poly(dA-dT)·poly(dA-dT) DNA, measured by the RNA polymerase reaction, was strongly inhibited; calf thymus DNA was less severely affected.

When cultures of Mucor hiemalis were incubated with [^{3}H]-dictamnine in the light, 0.2% of the administered label (0.18 µg·mg DNA^{-1}) was incorporated into the fungal DNA in vivo (38). The chemistry of the covalent adducts of dictamnine with nucleic acid bases has not been described. These furanoquinolines may have a role in protecting some plants against fungal attack, but this has yet to be demonstrated.

The furanochromones khellin (III) and visnagin, the active principals of the medicinal plant Ammi visnaga (41), are phototoxic towards gram-positive bacteria (32,42), viruses (43), and Saccharomyces and Candida (32). Khellin induces melanization in rabbit skin in sunlight (44), and causes gross chromosomal damage in CHO cells (32). Longwave UV irradiation of a frozen aqueous suspension of khellin and thymine resulted in the formation of a 2-2 adduct (IV) between the 2,3 double bond of khellin and the 5',6' double bond of thymine, indicating that the phototoxicity of khellin is due to a mechanism similar to the furanocoumarins. The incorporation of furanochromones into DNA in vivo has yet to be demonstrated. Some furanochromones, including khellin, are also known to be insect antifeedants (45), but as yet we can only speculate about the ecological significance of their phototoxic activity.

Similarly, the role of phototoxicity in the activity of the potent carcinogens, the aflatoxins (Va,b), produced by Aspergillus flavus and related species (46) is not apparent. Aflatoxins are phototoxic to Paramecium but not to E. coli (47), and have not yet been tested for phototoxicity against fungi. In the mammalian liver (and hence in the dark) they are converted to hepatocarcinogens when the double bond of the furan ring is epoxidized and the product subsequently forms covalent adducts to DNA (48). Excitation by UVA (365 nm) also induces the formation of adducts in vitro (49).

The β-carboline or harmane alkaloids (VI), the alkaloids 6-canthinone and 5-methoxy-6-canthinone (all from various Rutaceae) and the N-methyl substituted harmane brevicolline from the sedge Carex brevicollis are phototoxic to Saccharomyces and Candida (34). Structural similarity to the furanocoumarins suggests that they too may

photobind to DNA; this work is presently underway in our laboratory.

The isoflavonoid phytoalexins phaseollin, 3,6a,9- trihydroxyp-terocarpan, glyceollin, tuberosin, and pisatin, produced by several species of Fabaceae, photoinactivated glucose-6-phosphate dehydrogenase in an in vitro assay (50). Singlet oxygen quenchers, hydroxyl radical scavengers, and superoxide dismutase did not protect the enzyme against photoinactivation, ruling out a type II mechanism. ESR measurements confirmed the production of free radicals, which were most stable in the case of phaseollin. In the dark, glyceollin inhibits electron transport at some point beyond the succinate dehydrogenase site (51), and pisatin appears to uncouple oxidative phosphorylation (52). The aliphatic polyacetylenes increase membrane permeability and are highly toxic to Saccharomyces and other yeasts; their toxicity is not oxygen dependent and their rapid polymerization in UVA has been taken as evidence of free radical formation (53). Some chromenes and benzofurans, including encecalin (VII), are phototoxic to Saccharomyces and Candida (33). These compounds cause hemolysis of erythrocytes (54) indicating a target in the membrane, and may operate via a free radical mechanism.

Intermediate Photosensitizers

Many photosensitizers are capable of competing type I and type II reactions. Even 8-MOP, long considered a classical type I photosensitizer, generates singlet oxygen in the absence of suitable sites for photobinding (55). The relative significance of the two mechanisms is illustrated by the observation that E. coli mutants defective in the repair of oxidative damage are about 15 times less sensitive to 8-MOP induced damage than are excision repair deficient mutants, but are about 100 times more sensitive than wild type strains (56).

Competing type I and type II mechanisms are typical of the aromatic polyacetylenes, characteristic secondary metabolites of the Asteraceae and about twenty other families of vascular plants. Early work on the polyacetylene phenylheptatriyne (PHT) (VIII) showed reduced toxicity to Saccharomyces cerevisiae under aerobic conditions, and toxicity was not modified by azide (which quenches singlet oxygen) or D_2O (which increases the lifetime of singlet oxygen) (57). Low concentrations of PHT rapidly inhibited cell respiration, and did not increase sister chromatid exchanges (58), indicating that the nucleus was not a target; this compound thus did not resemble either the furanocoumarins or the photodynamic dyes in its mechanism of action. To complicate the story, it was subsequently found that PHT and other polyacetylenes are photodynamic towards some organisms, including E. coli, but are partially non-photodynamic in other systems, including Saccharomyces (53). With liposomes as a model membrane system, only the photodynamic activity could be demonstrated (59). The effect of PHT on membrane permeability was shown to depend on the degree of unsaturation of the membrane lipids. Permeability was greatly increased in liposomes composed of dipalmitoyl phosphatidylcholine (PC) and other saturated lipids, which present a highly ordered environment, and was increased to a lesser extent in disordered membranes composed of unsaturated lipids; in the latter case lipid peroxidation was shown

to be involved. The different effects of PHT in different organisms may be related to differing lipid environments in the membranes, but PHT also inactivates membrane-bound enzymes (60) and so the responses may also be related to accessibility of target proteins, possibly including respiratory centers.

Photochemical studies provide further information on competing type I and type II reactions with PHT. Laser excitation (308 or 337 mm) leads to the formation of a strong triplet signal, with a lifetime of 28 us in methanol, which was efficiently quenched by the triplet quencher 1,3 octadiene (61). Quenching with O_2 occurred with a rate constant comparable to the rate of electron transfer to methyl viologen. The formation of both singlet oxygen and the semioxidized PHT radical are consistent with the competing mechanisms observed in vivo. When irradiated with UVA, liposome bilayers composed of distearyl PC with PHT produced a free radical signal detected by electron spin resonance (ESR) spectroscopy (62). The spectrum consisted of one broad peak with a line width of 19G and a g value of 2.0017. The rate of formation was dependent on light intensity, PHT concentration within the bilayer, and concentration of the liposome–PHT suspension. The signal was enhanced in an anaerobic environment, indicating a non-oxidative mechanism for free radical formation. Once formed, the radical species was very stable in the presence of oxygen, decaying slowly over an 8-12 hour period. Formation of the radical was enhanced in an ordered lipid environment as incorporation of lysophosphatidyl-choline, which perturbs lipid packing, decreased levels of the free radical species. When PHT was present in a liposome with an even more fluid membrane, such as egg yolk PC, the levels of free radical generation were even lower.

Polyacetylene involvement in resistance to fungal attack is well documented, and includes the phytoalexins safynol and dehydrosafynol from Carthamnus tinictorius (63,64), wyerone from the broad bean, Vicia faba (65), and falcarinol, falcarindiol, and E-tetradeca-6-ene-1,3- diyne-5,8-diol from the tomato, Lycopersicon esculentum (66). These phytoalexins are toxic without photoactiva-tion, but their activity may, in some cases, be enhanced in UVA. Few studies specifically address the role of polyacetylene photo-sensitization in defense against fungal attack. PHT, present in the cuticle of Bidens pilosa leaves at concentrations up to 600 ppm, strongly inhibits the germination and growth of Fusarium culmorum in UVA but not in the dark; PHT was fungitoxic and not simply fungi-static (67). In this case the polyacetylene constitutes a preformed barrier against fungal attack. Nineteen species of phylloplane yeasts and yeast-like fungi, isolated from Hawaiian species of Bidens with and without leaf polyacetylenes, were tested for photo-sensitivity to those acetylenes (68). Although all these organisms, members of the Sporobolmycetaceae, Cryptococcaceae, and Fungi Imperfecti, were sensitive to some acetylenes and resistant to others, there was no correlation between the presence or absence of leaf polyacetylenes and the distribution of these saprophytes amongst the Bidens species, with one notable exception. The only pathogenic species isolated, Colletotrichum gloeosporiodes, did not colonize Bidens leaves containing C13 aromatic polyacetylenes, to which it is extremly sensitive in vitro.

Type II Photosensitizers

The classic type II sensitizers are the photodynamic dyes; the most active natural type II sensitizers yet discovered are the thiophenes, sulfur derivatives of the polyacetylenes. These compounds are restricted to advanced tribes of the Asteraceae, including the Vernonieae, Inuleae, Heliantheae, Anthemideae, Senecioneae, Cynareae, and particularly the Tageteae (69). Alpha-terthienyl (α-T) (IX) is toxic to bacteria, yeasts, and other fungi (11), and can photosensitize herbivorous insects (70,71). A strict requirement for oxygen in α-T toxicity has been demonstrated both in vivo (60,72-74) and in vitro (75-77). Although the specific cellular target involved has not been identified, it is certainly in the membrane as α-T is not photogenotoxic, and it increases membrane permeability and inactivates membrane-bound enzymes (60,74). The membrane-bounded viruses murine cytomegalovirus (CMV) and Sindbis virus were sensitive to α-T at 10 ug ml in UVA but not in the dark, but the membraneless virus T4 was much more resistant (78). Murine CMV which had been inactivated by α-T and UVA penetrated mouse cells efficiently but the viral DNA could not replicate and later viral proteins were not produced. As viral gene expression was inhibited it was suggested that α-T may interact with viral proteins as well as membrane lipids; α-T certainly oxidizes proteins in E. coli (74).

The thiophenes are involved in defense against fungal attack in Tagetes at least. Innoculation of Tagetes erecta with the pathogen Fusarium oxysporum elicits twelve-fold greater production of α-T, bithiophene hydroxyl, and bithiophene acetate (79). Two strains of the pathogen are known; the fast-growing virulent strain kills the plant before significant elevation of the thiophene levels can be accomplished, but the slower-growing strain is effectively combatted by these fungicidal compounds.

Recently we have described the antifungal activity of a group of acetylenic dithiacyclohexadienes, from Chaenactis douglasii and other members of the Asteraceae (80). These red compounds, christened thiarubrines (X), do not require light for their anti-fungal activity. However, when irradiated with UVA, their activity is enhanced and is then extended to bacteria and viruses. In light these unstable compounds lose one of the sulfur atoms of the ring and the resultant thiophene then displays the photodynamic activity characteristic of this class of phytochemicals. The dark antifungal activity remains unexplained.

The activity of the fungal photosensitizer cercosporin, a dihydroxyperylene quinone produced by various Cercospora species, is reviewed by Daub elsewhere in this volume. This apparently consti-tutes the first case in which a function other than defense can be ascribed to a phototoxin. As Cercospora hyphae do not penetrate the cells of the host plant, cercosporin-mediated lipoperoxidation of host membranes is essential to release nutrients required by the growing pathogen. Hypericin, a related compound found in most species of Hypericum, is also a photodynamic photosensitizer (81, P. Knox, this volume). The phototoxin occurs in glandular trichomes and is effective against insects, but its significance as a possible antifungal compound is unknown.
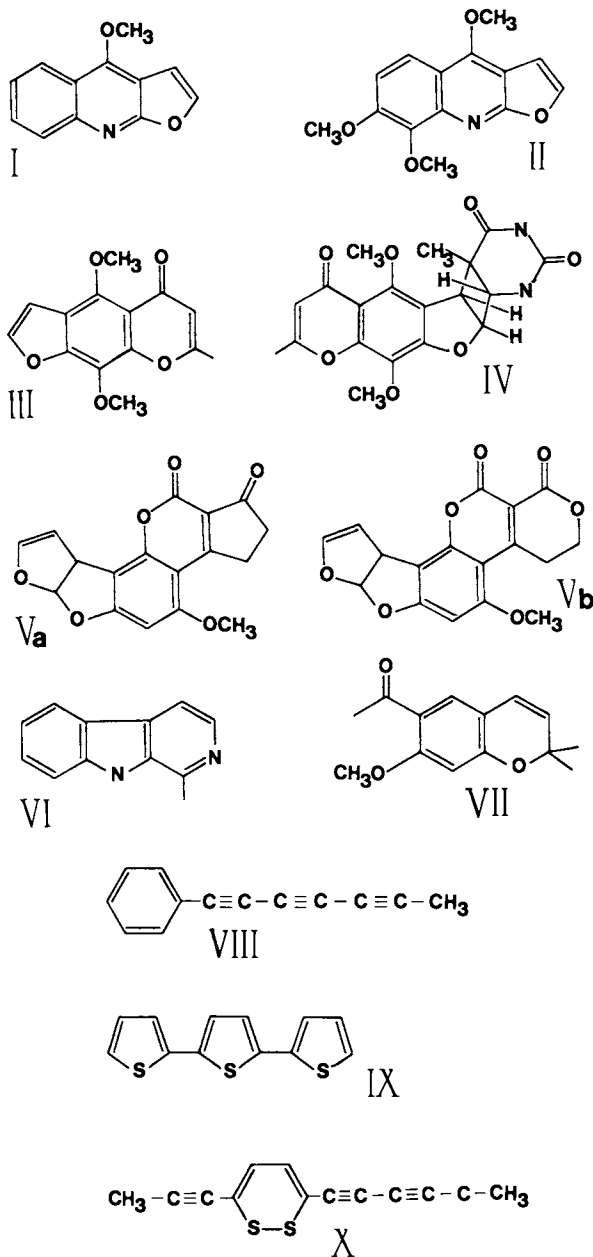
Structures I-X

Conclusion

The diversity of phototoxic compounds isolated from plants, fungi, and bacteria in recent years suggests that such compounds may be quite common in nature. Probably hundreds of such compounds remain to be identified. Much work remains to be done to identify the toxic mechanism and target sites of many of the photosensitizers which are currently known. The question of how organisms that produce phototoxins avoid autotoxicity has scarcely been addressed. Finally, the ecological significance of phototoxic secondary metabolites has only begun to be studied, but given the diversity of species which contain these compounds many fascinating interactions await description.

Literature Cited

1. Daniels, F. J. Invest. Dermatol. 1965, 44, 259-263
2. Spikes, J.D. In "The Science of Photobiology"; Smith, K.C., Ed.; Plenum Press: New York, 1977, pp 87-112
3. Krinsky, N.I. Photochem Photobiol. 1985, 41 (Suppl.), 96S
4. Downum, K.R. In "Natural Resistance of Plants to Pests"; Hedin, P.A., Ed.; ACS Symposium Series No. 296, American Chemical Society: Washington, D.C., 1986, pp. 197-205.
5. Ou, C.N.; Song, P.S. Biochemistry 1978, 17, 1054-1059.
6. Averbeck, D.; Moustacchi, E.; Bisagni, E. Biochem. Biophys. Acta 1978, 518, 464-481.
7. Veronese, F.M.; Schiavon, O.; Bevilacqua, R.; Bordin, F.; Rodighiero, G. Photochem Photobiol. 1982, 36, 25-30.
8. Granger, M.; Helene, C. Photochem Photobiol. 1983, 38, 563-568.
9. Dall'Acqua, F.; Marciani, G.; Ciavatta, L.; Rodighiero, G. Z. Naturforsch. 1971, 26, 561-569.
10. Song, P.S.; Tapley, K.J. Jr. Photochem Photobiol. 1979, 29, 1177-1197.
11. Towers, G.H.N. Can. J. Bot. 1984, 62, 2900-2911.
12. Averbeck, D.; Averbeck, S. Mutat. Res. 1978, 50, 195- 206.
13. Scott, R.B.; Alderson, T. Mutat. Res. 1971, 12, 29-31.
14. Averbeck, D.; Bisagni, E.; Marquet, J.P.; Vigny, P.; Garboriau, F. Photochem Photobiol. 1979, 30, 547-555.
15. Averbeck, D.; Moustacchi, E. Biochem. Biophys. Acta 1975, 395, 393-404.
16. Averbeck, D.; Moustacchi, E.; Bisagni, E. Biochem. Biophys. Acta 1978, 518, 464-481.
17. Alderson, T.; Scott, B.R. Mutat. Res. 1970, 9, 569-578.
18. Chackraborty, D.P.; Das Gupta, A.; Bose, P.K. Ann. Biochem. Exp. Med. 1957, 17, 59-62.
19. Mikkelson, V.E.; Fowlks, E.W.; Griffith, D.G. Arch. Phys. Med. 1961, 42, 609-613.
20. Muronets, E.M.; Kovtumemko, L.V.; Kameneva, S.V. Genetika (Moscow) 1980, 16, 1168-1175.
21. Musajo, J.F.; Bordin, F.; Bevilacqua, R. Photochem. Photobiol. 1967, 6, 927-931.

22. Musajo, J.F.; Bordin, F,; Caporale, G.; Marciani, S.; Rigatti, G. Photochem. Photobiol. 1967, 6, 711-719.
23. Cole, R.S. Biochem. Biophys. Acta 1970, 217, 30-39.
24. Cole, R.S. J. Bacteriol. 1971, 107, 846-852.
25. Chandra, P.; Kraft, S.; Wacker, A.; Rodighiero, G.; Dall'Acqua, F.; Marciani, S. Biophysik. 1971, 7, 251- 258.
26. Marciani, S.; Terbojevich, M.; Dall'Aqcqua, F. Z. Naturforsch. 1972, 27b, 196-200.
27. Musajo, L.; Rodighiero, G. In "Photophysiology, Vol. VII"; Geise, A.C., Ed.; Academic: New York, 1972, pp. 115-147.
28. Johnson, C.; Brannon, D.R. Phytochem. 1973, 12, 2961.   29.
    Beier, R.C.; Oertli, E.H. Phytochem. 1983, 22, 2595.
30. Chandhary, S.K.; Ceska, O.; Warrington, P.J.; Ashwood-Smith, M.J. J. Agric. Food Chem. 1985, 33, 1153.
31. Berenbaum, M.R. Science 1978, 201, 532-534.
32. Abeysekera, B.F.; Abramowski, Z.; Towers, G.H.N. Photochem. Photobiol. 1983, 38, 311-315.
33. Proksch, P.; Proksch, M.; Towers, G.H.N.; Rodriguez, E. J. Nat. Prod. 1983, 46, 331-335.
34. Towers, G.H.N.; Abramowski, Z. J. Nat. Prod. 1983, 46, 576-581.
35. McKenna, D.J.; Towers, G.H.N. Phytochem. 1981, 20. 1001-1004.
36. Towers, G.H.N.; Graham, E.A.; Spenser, I.D.; Abramowski, Z. Planta Medica 1981, 41, 136-142.
37. Murray, R.D.H.; Mendez, J.; Brown, S.A. "The Natural Coumarins: Occurrence, Chemistry, and Biochemistry"; Wiley: New York, 1982.
38. Pfyffer, G.E.; Towers, G.H.N. Can. J. Microbiol. 1982, 28, 468-473.
39. Philogene, B.J.R.; Arnason, J.T.; Towers, G.H.N.; Abramowski, Z.; Campos, F.; Champagne, D.; McLachlan, D. J. Chem. Ecol. 1984, 10, 115-123.
40. Pfyffer, G.A.; Pfyffer, B.U.; Towers, G.H.N. Photochem. Photobiol, 1982, 35, 793-797.
41. Schonberg, A.; Sina, A.J. J. Am. Chem. Soc. 1950, 72, 1611.
42. Fowlks, W.L.; Griffith, D.G.; Oginsky, E.L. Nature 1958, 181, 571-572.
43. Cassuto, E.; Gross, N.; Bardwell, E.; Howard-Flanders, P. Biochem. Biophys. Acta 1977, 475, 589-600.
44. Kabilov, N.M. Farmakol. i. Tokisol. 1962, 25, 733-735.
45. Yajima, T.; Munakata, K. Agric. Biol. Chem. 1979, 43, 1701-1706.
46. Diener, U.L.; Davis, N.D.; In "Aflatoxins"; Goldblatt, L.A., Ed.; Academic Press: New York, 1969, pp. 13-54.
47. Smith, R.C.; Neely, W.C. Can. J. Microbiol. 1972, 18, 1965-1967.
48. Wogan, G.N. Ann. Rev. Pharmacol. 1975, 15, 437-453.
49. Sheih, J.-C.; Song, P.-S. Cancer Res. 1980, 40, 689-
50. Bakker, J.; Gommers, F.J.; Smits, L.; Fuchs, A.; de Vries, F.W. Photochem. Photobiol. 1983, 38, 323-330.
51. Kaplan, D.T.; Keen, N.T.; Thomason, I.J. Physiol. Plant Pathol. 1980, 16, 319-325.
52. Oku, H.; Ouchi, S.; Shiraishi, T.; Utsumi, K.; Seno, S. Proc. Jpn. Acad. 1976, 52, 33-36.
53. McLachlan, D.; Arnason, J.T.; Lam, J. Photochem. Photobiol. 1984, 39, 177-182.
54. Aregullin-Gallardo, M. Ph.D. Thesis, University of California, Irvine, 1985.

55. Vedaldi, D.; Dall Acqua, F.; Gennaro, A.; Rodighiero, R. Z. Naturforsch. 1983, 38, 866-869.
56. Tuveson, R.W.; Berenbaum, M.R.; Heininger, E.E. J. Chem. Ecol. 1986, 12, 933-948.
57. Arnason, J.T.; Wat, C.-K.; Downum, K.; Yamamoto, E.; Graham, E.; Towers, G.H.N. Can. J. Microbiol. 1980 26, 698-705.
58. MacRae, W.D.; Chan, G.F.Q.; Wat, C.-K.; Towers, G.H.N.; Lam, J. Experientia 1980, 36, 1096-1097.
59. McRae, D.G.; Yamamoto, E.; Towers, G.H.N. Biochem. Biophys. Acta 1985, 821, 488-496.
60. Yamamoto, E.; Wat, C.-K.; MacRae, W.D.; Towers, G.H.N. FEBS Letters 1979, 107, 134-136.
61. Weir, D.; Scaiano, J.C.; Arnason, J.T.; Evans, C. Photochem. Photobiol. 1984, 42, 223-230.
62. McRae, D.; Yamamoto, E.; Towers, G.H.N. (unpublished results).
63. Allen, E.; Thomas, C. Phytochem. 1971, 10, 1579-1582.
64. Allen, E.; Thomas, C. Phytopathol. 1971, 61 1107- 1109.
65. Hargraves, J.A.; Mansfield, J.W.; Coxon, D.T.; Price, K.R. Phytochem. 1976, 15, 1119-1121.
66. DeWit, P.J.G.M.; Koddle, E. Physiol. Plant Pathol. 1981, 18, 143-148.
67. Bourque, G.; Arnason, J.T.; Madhosingh, C.; Orr, W. Can. J. Bot. 1985, 63, 899-902.
68. Marchant, Y.Y.; Towers, G.H.N. Biochem. System. Ecol. (in press).
69. Bohlman, F.; Burkhardt, T.; Zdero, C. "Naturally Occurring Acetylenes"; Academic Press: New York, 1973, 547 p.
70. Downum, K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest. Biochem. Physiol. 1984, 22, 104-109.
71. Champagne, D.E.; Arnason, J.T.; Philogene, B.J.R.; Morand, P. Lam, J. J. Chem. Ecol. 1986, 12, 835-858.
72. Arnason, J.T.; Chan, G.F.Q.; Wat, C.-K.; Downum, K.; Towers, G.H.N. Photochem. Photobiol. 1981, 33, 821- 824.
73. Gommers, F.J.; Bakker, J.; Wynberg, H. Photochem. Photobiol. 1982, 35, 615-619.
74. Downum, K.R.; Hancock, R.E.W.; Towers, G.H.N. Photochem. Photobiol. 1982, 36, 517-524.
75. Wat, C.-K.; MacRae, W.D.; Yamamoto, E.; Towers, G.H.N.; Lam, J. Photochem. Photobiol. 1980, 32, 167-172.
76. Reyftmann, J.P.; Kagan, J.; Santus, R.; Marliere, P. Photochem. Photobiol. 1985, 41, 1-7.
77. Evans, C.; Weir, D.; Scaiano, J.C.; MacEachern, A.; Arnason, J.T.; Morand, P.; Hollebone, B.; Leitch, L.C.; Philogene, B.J.R. Photochem. Photobiol. (in press).
78. Hudson, J.B.; Graham, E.A.; Micki, N.; Hudson, L.; Towers, G.H.N. Photochem. Photobiol. (in press).
79. Kourany, E. M.Sc. Thesis, University of Ottawa, 1986. 80.
80. Towers, G.H.N.; Abramowski, Z.; Finlayson, A.J.; Zucconi, A. Planta Medica 1985, XX, 225-229.
81. Knox, J.P.; Dodge, A.D. Plant, Cell, Environ. 1985, 8, 19-25.

Chapter 17

Structure and Function Relationships in Polyacetylene Photoactivity

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Polyacetylenes and thiophenes have light-independent and
photoactivated toxic effects on a wide range of organisms.
These biologically active compounds are of academic and
practical interest because of their possible role as plant
defense compounds and their potential as commercially useful
biocidal agents. Many factors influence the extent of damage
caused by these compounds and this paper reviews the
relationship between structure and function in polyacetylenes
and presents data in support of current views.

Polyacetylenes and their thiophene derivatives are biologically
active secondary metabolites characteristic of taxonomically
advanced plant families such as the Asteraceae, the Apiaceae, the
Araliaceae and the Campanulaceae, as well as certain groups of
Basidiomycete fungi (1-5). Only seven compounds were described
between 1902, when Arnaud first established the existence of a
naturally-occurring triple bond (6), and 1950, when antibiotic
substances produced by fungal species were identified as acetylenes
(7-12; Figure 1). Since then several hundred polyacetylenes have
been recorded (3), many with toxic activity against biological
systems, because investigations into the antibiotic properties of
plants and fungi often led to the discovery of polyacetylenes as the
active principles. Identification was facilitated by the
characteristic UV spectra of conjugated acetylenes and by the high
extinction coefficients of the spectra which permitted detection of
low quantities of compounds in extracts (1,13,14).
    In 1973 the nematocidal properties of alpha-terthienyl (II) and
5-(3-buten-1-ynyl)-2,2'-bithienyl (III; Figure 2) were found to be
significantly enhanced by UV light (15). This led to a systematic
investigation of the phototoxic properties of polyacetylenes from
the Asteraceae by Towers and his associates (5, 16-19). The in
vitro photoactivity of acetylenic compounds against biological
systems is now a well established phenomenon. Nevertheless, many
biologically active acetylenes are not light-activated and must be
considered in any attempt to understand the structural basis for the
phototoxicity of these compounds. This paper will examine the

relationship between structure and biological activity in polyacetylenes.

Polyacetylenes with Light-Independent Biological Effects

Bohlmann et al. (1) surveyed 32 species in the Campanulaceae and found that the most commonly occurring acetylenes in this family are the C_{14}-ene-diyne-ene tetrahydropyranylethers (IV; Figure 3), none of which appear to be photosensitizers, or even to have antibiotic activity (5). The characteristic compounds of the Apiaceae and the Araliaceae, falcarinone and falcarindiol (V, VI; Table I), are

Table I. Biologically active C_{17} acetylenes
from the Apiaceae and Araliaceae

$$CH_2=CH-\overset{\overset{\text{O}}{\|}}{C}-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_6-CH_3$$

V

$$CH_2=CH-\overset{\overset{\text{OH}}{|}}{C}-(C\equiv C)_2-CH_2-\overset{}{C}H-CH-(CH_2)_6-CH_3$$

VI   OH

$$CH_3-(CH_2)_2-\overset{\overset{\text{OH}}{|}}{C}H-(CH_2)_2-(CH=CH)_2-(C\equiv C)_2-CH=CH-(CH_2)_2OH$$

VII

$$CH_3-(CH_2)_2-\overset{}{C}H-(CH=CH)_3-(C\equiv C)_2-CH_2CH_2CH_2OH$$
                              OH   **VIII**
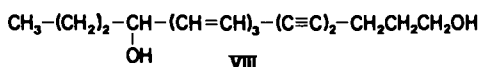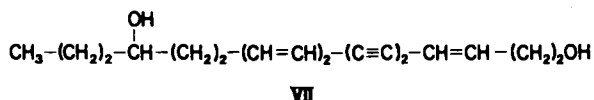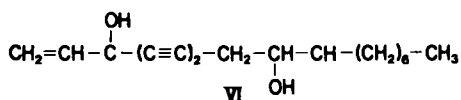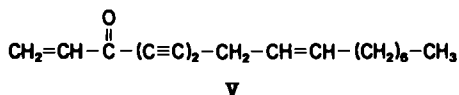
Falcarinone (V), falcarindiol (VI), Daucus carota L.; oenanthotoxin (VII), Oenanthe crocata L.; cicutotoxin (VIII), Cicuta virosa L.

active against pathogenic and dermatophytic fungi (20-27), insects such as Daphnia magna Straub (28), cause erythrocyte hemolysis (21,29), and have various pharmacological effects (30), all of which are light independent. Effective concentrations differ for each experimental system, but appear to be within the range of 10^{-5} to 10^{-4} M. Two other C_{17} polyacetylenes, oenanthotoxin (VII) and cicutotoxin (VIII) from Oenanthe crocata L. and Cicuta virosa L., are much more potent compounds, with well documented fatal neurotoxic effects on livestock and humans (31).
    Acetylenes from the Asteraceae also have numerous non-photoactivated biological effects (Table II). For example, the

$$H(C{\equiv}C)_2-CH{=}C{=}CH-(CH{=}CH)_2-CH_2COOH$$
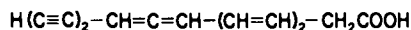
I

Figure 1. The first fungal antibiotic acetylene isolated
from Basidiomycetes. Mycomycin (I), <u>Nocardia</u> <u>acidophilus</u>.

II

$$C{\equiv}C-CH{=}CH_2$$
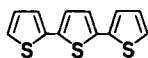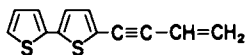
III

Figure 2. Photoactivated nematicidal thiophenes from the
Asteraceae. <u>Alpha</u>-terthienyl (II) ; 5-(3-buten-1-ynyl)-2,2'
bithienyl (III), <u>Tagetes</u> <u>patula</u> L.

$$HOCH_2-CH{=}CH-(C{\equiv}C)_2-CH{=}CH$$
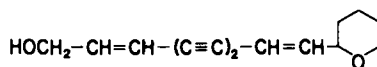
IV

Figure 3. Typical polyacetylene from the Campanulaceae.
C_{14}-tetrahydropyranylether (IV), <u>Campanula</u> spp.

Table II. Non-photoactivated polyacetylenes from the Asteraceae

CH₃—(C≡C)₃—CH=CH— [RO-tetrahydropyran]

$CH_3-(C\equiv C)_3-CH=CH-$

R = H   IX
R = CH₃CO   X

[phenyl]—C(=O)—(C≡C)₂—CH₃

XI

        CH₃
        |
HC≡C—C—C≡CH
        |
    [phenyl]

XII

                                    O
                                    ||
CH₃—CH=CH—(C≡C)₂—CH=CH—COCH₃
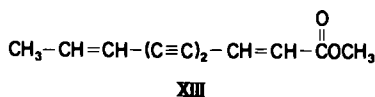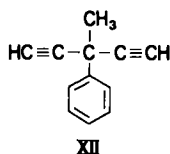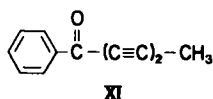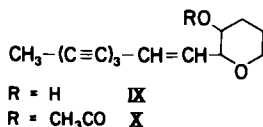
XIII

Ichthyothereol (IX), ichtyothereol acetate (X), Ichthyothere
terminalis Spreng.; capillin (XI), Artemisa capillaris Thunb.; 3-
methyl-3-phenyl-1,4-pentadiyne (XII), Artemesia monosperma Delile;
matricaria ester (XIII), Solidago altissima L.

active principles in the plants used as fish poisons in parts of
South America (Ichthyothere and Clibadium spp.) are the C14-ene-
triyne-tetrahydropyranes ichthyothereol and its acetate (IX, X; 32),
which differ from the inactive compounds of Campanula by the
presence of one extra alkynyl group. Capillin (XI), a conjugated
acetylenic ketone from Artemesia capillaris Thunb., has antifungal
and anti-inflammatory properties, and is active against dermal
mycoses (33-35). Artemesia monosperma Delile, an Egyptian desert
medicinal herb, is not attacked by insects (36). Its aerial
portions contain an aromatic diacetylene 3-methyl-3-phenyl-1,4-
pentadiyne (XII) which has potent insecticidal effects (37).
Matricaria ester (XIII) and its derivatives not only have
antifeedant effects upon phytophagous insects (38), they inhibit
seedling germination in vitro (39). Several other acetylenes have
been reported to be phytotoxic (40,41) although it remains unclear

whether these compounds are actually released into the rhizosphere
where allelopathic interactions are thought to occur (5,42).

Structure/Function Relationships in Polyacetylene Photoactivity

Prior to the discovery of polyacetylene phototoxicity, Reisch et al
(43) had investigated the bacteriostatic and fungistatic effects of
a large number of simple synthetic acetylenes, including
hydrocarbons, acids, alcohols, aldehydes and ketones with one or two
triple bonds, as well as the C_{13}-ene-tetrayne and pentayne-ene
compounds (XIV, XV; Table III). Their findings suggest that

Table III. Photoactivated polyacetylenes from the Asteraceae

$$CH_2=CH-(C\equiv C)_4-CH=CH-CH_3$$

XIV

$$CH_2=CH-(C\equiv C)_5-CH_3$$

XV

$$CH_3-CH=CH-(C\equiv C)_3-CH=CH-\overset{OH}{\underset{|}{CH}}-CH_2OH$$

XVI

$$CH_3-(C\equiv C)_3-CH=CH-\langle furan \rangle$$

XVII

XVIII

$$CH_3-CH=CH-(C\equiv C)_3-CH=CH-\overset{Cl}{\underset{|}{CH}}-CH_2OH$$

XIX

$$\langle phenyl \rangle-(C\equiv C)_3-CH_3$$

XX
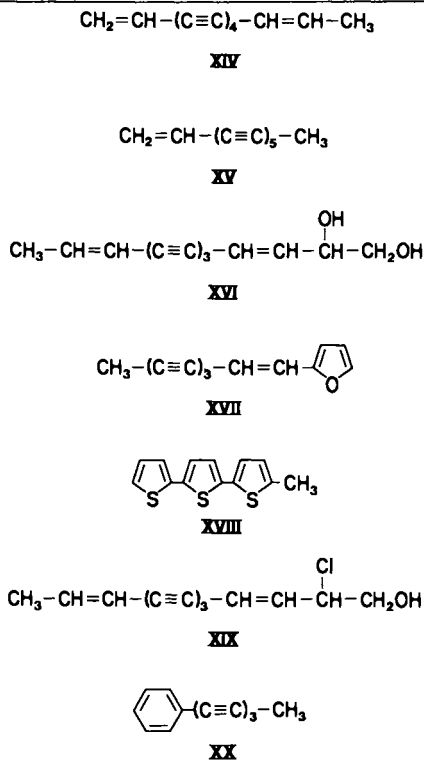
C_{13}-1,11-diene-3,5,7,9-tetrayne (XIV), C13-1-ene-3,5,7,9,11-
pentayne, <u>Bidens</u>, <u>Coreopsis</u> spp.; safynol (XVI), <u>Carthamus</u> <u>tinctoria</u>
L.; C$_{13}$-5-ene-7,9,11-triyne-furan (XVII), <u>Chrysanthemum</u> <u>leucanthemum</u>
L.; 2-methyl thiophene (XVIII), <u>Tagetes</u> spp.; C13-3,11-diene-
5,7,9,triyne-2-chloro-1-ol (XIX), <u>Centaurea</u> <u>ruthenica</u> Lam.; 1-
phenylhepta-1,3,5,-triyne (XX) <u>Bidens</u> <u>alba</u> L.

acetylenes with aromatic substituents are most active and that
fungicidal effects increase with the degree of unsaturation in the
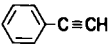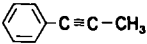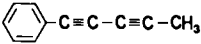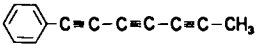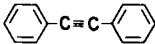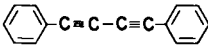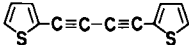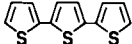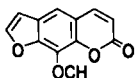
molecule and polarization of the triple bond, while compounds which
are more hydrophilic tend to be bacteriocidal agents. As distinct
from the mainly aliphatic acetylenes of the other families,
acetylenes produced by the Asteraceae are characterized by highly
unsaturated hydrocarbons and cyclic, aromatic or heterocyclic groups
(1). Some of these complex structures are restricted in
distribution while compounds such as thiophenes have been found in
the majority of tribes, their occurrence seemingly unrelated to
other taxonomic characters (3). Significantly photoactive
polyacetylenes occur only in the Asteraceae.

More than two dozen compounds from the Asteraceae have been
extensively tested for photoactivity against various biological
systems. In general, aliphatic compounds containing fewer than
three conjugated acetylenic bonds do not exhibit phototoxic effects
against yeasts, filamentous fungi, Gram-negative bacteria, nematodes
or mosquito and blackfly larvae (16,44-47) although not all
compounds with three or more triple bonds are photoactive, nor is
photoactivity uniformly expressed against all organisms. Safynol
(XVI) is active against microorganisms and mosquito larvae (44, 16)
but IX is only phototoxic to nematodes (45). Extracts from
Grindelia species were not photoactive or antibiotic to Candida
albicans (Robin) Berkh. (19) probably because none of the acetylenes
isolated from this genus contain more than two triple bonds (1).
Similarly, an extensive survey of 80 Asteraceae species for UV-
mediated activity by Camm et al. (17) reveals that only those which
produce furanoacetylenes (Erigeron spp.), thiarubrines and
thiophenes (Tagetes, Heliopsis, Rudbeckia spp.), and aliphatic
compounds with four or five conjugated triple bonds (Arnica,
Centaurea spp.) (1) exhibit phototoxicity to C. albicans. The
photoactivity of aromatic and highly unsaturated acetylenes from
Bidens and Coreopsis species has also been well documented (48, 49).

A recent report by Arnason et al. (46) showed that
furanoacetylenes require three conjugated triple bonds (XVII) for
optimal phototoxicity against Aedes aegypti larvae, and that
although methyl- and benzyl-substituted derivatives of alpha-
terthienyl (II) had absorption spectra similar to II, only 2-methyl-
thiophene (XVIII) was more active. In another study, the relative
toxicity of a series of chemically related polyacetylenes was
quantitatively evaluated for activity against Saccharomyces
cerevisiae and Escherichia coli (47). The organisms exhibited
differential photosensitivity to some of the compounds but, in
general, thiophenes were more toxic than aromatic acetylenes and
straight chain hydrocarbons were least active except for a
chlorinated ene-triyne-ene alcohol (XIX) whose effects were
comparable to that of the thiophenes. It is noteworthy that XIX is
not active against mosquito larvae (16).

The relationship between chemical structure, UV absorption and
degree of phototoxicity was examined using another series of
naturally-occurring and synthetic acetylenes (Table IV).
Phenylacetylene (XXI), phenylpropyne (XXII), diphenylacetylene
(XXIV), dithiophene (XXVII) and 8-methoxypsoralen (XVIII) were
purchased from Aldrich Chemicals. Compounds II, XX, XXIII, XXV and
XXVI were synthesized according to published methods (50-52). 8-
Methoxypsoralen was used as a reference photoactive compound (53) in

Table IV. Naturally occurring and synthetic polyacetylenes and thiophenes tested for phototoxicity against microorganisms

Phenylacetylene (XXI); phenylpropyne (XXII), phenylpentadiyne (XXIII), phenylheptatriyne (XX), diphenylacetylene (XXIV), diphenylbutadiyne (XXV), alpha-bithienyl (XXVI), dithienyl butadiyne (XXVII), alpha terthienyl (II); 8-methoxypsoralen (XXVIII), linear furanocoumarin used as reference compound.

the modified disc bioassay of Daniels (54) described elsewhere (49). Two yeasts and six bacterial species were used as test organisms, including DNA-repair-deficient mutant E. coli B$_{(s-1)}$(rec+, exr-, hrc-) which is extremely sensitive to UV radiation and to chemical alkylating agents (55,56) (Table V). The two Pseudomonas species are photoinsensitive and served as controls. All microorganisms were provided by Professor G.H.N. Towers, University of British Columbia.

In the standard bioassay, 10 μg of each compound is administered and allowed to diffuse into the agar for 30 minutes, then test plates are irradiated for 2 hours with longwave UV lamps (320-400nm) of 1.6-2.0Wm^{-2} intensity at 15cm. Control plates are kept in the dark. Toxicity is measured by the size of the inhibition zones in the culture lawns. Diffusion effects in this
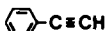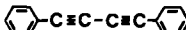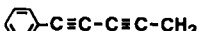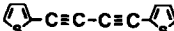
series is minimal because of the similar molecular weights and polarity characteristics of the compounds. Results are shown in Table VI. In aromatic acetylenes at least two conjugated triple

Table V. Microorganisms used in phototoxicity assays.

Escherichia coli B/r
E. coli B(s-1)
Pseudomonas aeroginosa
P. fluorescens
Staphylococcus albus
Streptococcus faecalis
Bacillus subtilis
Candida utilis
Saccharomyces cerevisiae

Table VI. Phototoxicity of polyacetylenes and thiophenes against microorganisms under standard aerobic conditions

Test Compounds \ Organisms	E. coli B/r	E. coli B(s-1)	P. aeroginosa	P. fluorescens	S. albus	B. subtilis	C. utilis	S. cerevisiae
8-METHOXY PSORALEN	++	+++	-	-	+++	++	++	+++
⬡-C≡CH	-	-	-	-	-	-	-	-
⬡-C≡C-⬡	-	-	-	-	-	-	-	-
⬡-C≡C-C≡C-⬡	++	-	-	-	+++	+	+++	++
⬡-C≡C-CH$_3$	-	-	-	-	-	-	-	-
⬡-C≡C-C≡C-CH$_3$	++	++	-	-	+++	+++	+++	++
⬡-C≡C-C≡C-C≡C-CH$_3$	++++	+++	-	-	++++	++++	++++	++++
(thiophene dimer)	++	-	-	-	+++	+	++	+
(thiophene trimer)	+++	++	-	-	++++	+	+++	+++
(thiophene)-C≡C-C≡C-(thiophene)			-	-		++++	++++/IA	++++

Diameters of clear zones: + 8-12mm.; ++ 12-18mm.; +++ 18-30mm.; ++++ > 30mm.

bonds are necessary for photoactivity. An inspection of the data indicates that photoactivity is directly correlated with the number of acetylenes in the molecule. The monoacetylenes do not have strong absorption in the longwave UV region (Figure 4) which suggests poor efficiency of photon capture in that range. Among the thiophenes, II is more potent than XXVI but not as effective as XXVII, and dithienylbutadiyne (XXVII) is more toxic than diphenylbutadiyne (XXV), which supports other observations that the thiophene moiety has some particularly effective biocidal characteristics (5,44). The spectrum of XXVII has a substantial longwave component (about 360nm) whereas that of XXV does not extend much beyond 320nm (Figure 4). If phototoxicity depends on the number of photons absorbed by the sensitizing molecule and the UV absorption of a molecule does not correspond to the emmission spectrum of the longwave UV source, it would not be expected to have significant phototoxic properties. McLachlan et al. (47) have reported that this is not always true. Some polyacetylenes (XIX and XX) have relatively low energy absorption values in the 320-400nm range yet are comparable to thiophenes in toxicity while other compounds with spectra similar to II have no toxicity at all. It is obvious that in vitro studies have their limitations and other factors, not easily measured, must be important in such complex biological interactions.

Polyacetylene Structure and Mechanisms of Action

Unlike the linear furanocoumarins, e.g. 8-methoxypsoralen (XXVIII), which kill cells by a photoinduced modification of DNA (57), photoactive polyacetylenes and thiophenes attack cell membranes (29,58-61) by photodynamic as well as oxygen-independent mechanisms (62-68). In general, straight chain aliphatic acetylenes such as XIV, XV and XIX, which are notoriously unstable in vitro, have a non-oxidative mode of action which probably involves the formation of free radicals upon photoexcitation (62,66). Thiophenes, however, are Type II photodynamic photosensitizers which damage membranes via the catalytic generation of singlet oxygen (58,63,64,66). Partly cyclized aromatic acetylenes such as phenylheptatriyne (XX) which are intermediate in structure between the aliphatic compounds and the thiophenes apparently exhibit both photodynamic and nonphotodynamic processes (66,67). Most acetylenes are able to produce singlet oxygen in vitro at levels which do not fully account for their phototoxic effects, and in oxygen removal experiments, phenylacetylenes showed only partial or no decrease in phototoxicity to microorganisms (66) or photohemolysis of erythrocytes (29).
    In addition to the standard phototoxicity assay which is done under aerobic conditions (49,53), the polyacetylene series shown in Table IV was tested against seven bacteria and S. cerevisiae in the absence of oxygen. Materials and the protocol described for BBL GasPak Anaerobic Systems were obtained from BBL Microbiology Systems, P.O.Box 243, Cockeysville MD 21030. Procedures for the anaerobic assays were identical to that described above except that steps were taken to ensure the virtual absence of oxygen from the experimental system. All media were prereduced in sealed jars and all technical manipulations were carried out in a bag flushed
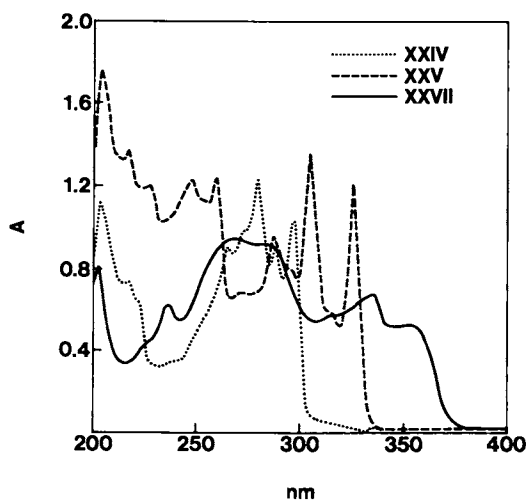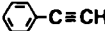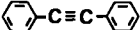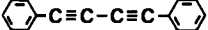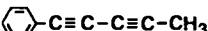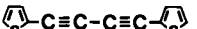
Figure 4. UV-absorption spectra of diphenylacetylene (XXIV), diphenylbutadiyne (XXVII), and dithienylbutadiyne (XXVII).

continuously with nitrogen gas. After the plates were streaked and the compounds applied, they were sealed in anaerobic pouches transparent to UV and the test plates irradiated. The organisms were then allowed to grow aerobically for 24 or 48 hours. Results are shown in Table VII and support the data previously reported by

Table VII. Phototoxicity of polyacetylenes and thiophenes against microorganisms under anaerobic conditions

Test Compounds	E. coli B/r	E. coli B(s-1)	P. aeruginosa	P. fluorescens	S. albus	S. faecalis	B. subtilis	S. cerevisiae
8-METHOXY PSORALEN	+++	+	−	−	+++	+	++	++
⬡–C≡CH	−	−	−	−	−	−	−	−
⬡–C≡C–⬡	−	−	−	−	~	−	−	−
⬡–C≡C–C≡C–⬡	−	+++	−	−	++	−	+	+++
⬡–C≡C–CH₃	−	−	−	−	−	−−	−	−
⬡–C≡C–C≡C–CH₃	−	+++	−	−	+++	+	+	++
⬡–C≡C–C≡C–C≡C–CH₃	−	+++	−	−	++++	++++	++	++
bithiophene								
terthiophene	−		−	−	−	−	−	−
thiophene–C≡C–C≡C–thiophene	++	++++	−	−	++++		+++	+++

Diameters of clear zones: + 8-12mm.; ++ 12-18mm.; +++ 18-30mm.; ++++ >30mm.

others (62-68). The aromatic acetylenes XX, XXIII and XX were active under aerobic and anaerobic conditions whereas alpha-terthienyl (II) clearly requires the presence of oxygen to be effective. Compound XXVII which is the thiophene analogue of diphenylbutadiyne (XXV), also operates via both types of mechanisms although it is more toxic than the latter.

Conclusions

Polyacetylenes and thiophenes have both light-independent and
photoactive effects on a wide range of biological systems. There is
a substantial body of evidence which shows that compounds containing
at least two conjugated carbon-carbon triple bonds can kill cells or
inhibit growth by damaging cellular membranes. Photoactivated
acetylenes are of particular interest because of their potential as
commercially useful and environmentally non-threatening biocidal
agents. Therefore, the relationship between structure and function
must be explored, although many complex and often unmeasurable
factors play a role in biological interactions and must be taken
into consideration in such studies. Many details are known about
polyacetylenes and their in vitro effects, but is must be noted that
although there is some circumstantial evidence and considerable
speculation about their in vivo functions, there is as yet no clear
understanding of their putative role in nature and, at present, no
obvious physiological role can be assigned to polyacetylenes in the
plants which produce them.

Acknowledgments

Literature Cited

1. Bohlmann, F.; Burkhardt, T.; Zdero, C. Naturally Occurring
    Acetylenes; Academic Press, London, 1973.
2. Hansen, L.; Boll, P.M. Phytochemistry 1986, 25, 285-293.
3. Sorensen, N.A. In The Biology and Chemistry of the Compositae;
    Vol I, Heywood, V.H., Harborne, J.B., Turner, B.L., Eds.;
    Academic Press, New York, 1977; pp 385-409.
4. Thaller, V. Royal Soc. Chem. Aliph. Nat. Prod. Chem. 1976-
    1977, 1, 1-19.
5. Towers, G.H.N. Can. J. Bot. 1984, 62, 2900-2911.
6. Arnaud, A. C.R. Hebd. Seanc. Acad. Sci.; Paris, 1902, 134, 473-
    482.
7. Anchel,. M. Am. Chem. Soc. J. 1953, 75, 421-462.
8. Anchel, M.; Polatnick, J.; Kavanagh, F. Arch. Biochem. 1950,
    25, 208-220.
9. Celmer, W.D.; Solomon, I.A. Amer. Chem. Soc. J. 1952a, 74,
    1870-1871.
10. Celmer, W.D.; Solomon, I.A., Amer. Chem. Soc. J. 1952b, 74,
    2245-2248.
11. Celmer, W.D.;Solomon, I.A., Amer. Chem. Soc. J. 1953, 75, 1372-
    1376.
12. Kavanagh, F.; Hervey, A.; Robbins, W.J. Proc. Nat. Acad. Sci.
    1950, 36, 102-106.
13. Jones, E.R.H. Pedlar Lecture, Feb 1959, Chemical Society,
    London.
14. Jones, E.R.H. Chem. Brit. 1966, 1966, 6-13.
15. Gommers, F.J.; Geerligs, J.W.G. Nematologia 1973, 19, 389-393.

16. Arnason, J.T.; Swain, T.;Wat, C.K.; Graham, E.A.; Partington, S.; Towers, G.H.N.; Biochem.Syst. Ecol. 1981, 9, 63-68.

17. Camm, E. L. ;Towers, G.H.N.; Mitchell. J.C. Phytochemistry 1975, 14, 2007-2011.

18. Towers, G.H.N.; Wat, C.K.; Graham, E.A.; Bandoni, R.J.; Chan, G.F.Q.; Mitchell, J.C.; Lam, J. Lloydia 1977, 40, 487-496.

19. Wat, C.K.; Johns, T. ; Towers, G.H.N. J. Ethnopharm. 1980, 2, 279-290 .

20. De Wit, P.J.; Kodde, E. Physiol. Plant Path. 1981, 18, 143-148.

21. Garrod, B.; Lea, E.J.A.; Lewis, B.G. New Phytol. 1979, 83, 463-472.

22. Garrod, B.; Lewis, B.G. Trans. Br. Mycol. Soc. 1982, 78, 533-536.

23. Harding, V.K.; Heale, J.B. Physiol. Plant Path. 1980, 17, 277-289.

24. Harding, V.K.; Heale, J.B. Physiol. Plant Path. 1981, 18, 7-15.

25. Kemp, M.S. Phytochemistry 1978, 17, 1002.

26. Muir, A.D.; Walker, J.R.L. Chem. N.Z. 1979, 43, 94-95.

27. Muir, A.D.; Cole, J.L.; Walker, J.R.L. Planta Med. 1982, 44, 129-133.

28. Crosby, D.G.; Aharonson, N. Tetrahedron 1967, 23, 465-472.

29. Wat, C.K.; MacRae, W.D.; Yamamoto, E.; Towers, G.H.N.; Lam, J. Photochem. Photobiol. 1980, 32, 167-172.

30. Tanaka, S.; Ikeshiro, Y. Arzneim. Forsch. /Drug Res. 1977, 27, 2039-2045.

31. Anet, E.; Lythgoe, B.; Silk, M.H.; Trippett, S. J. Chem. Soc. 1953, 1953, 309-322.

32. Cascon, S.C.; Mors, W.B.; Tursch, R.T.; Aplin, R.T.; Durham, L. J. Amer. Chem. Soc. J. 1965, 87, 5237- 5241.

33. Jones, E.R.H.; Thaller, V. In The Chemistry of the Carbon-Carbon Triple Bond Part 2, Patai, S., Ed.; J.Wiley and Sons, New York, 1978, pp 621-633.

34. Wagner, H. In The Biology and Chemistry of the Compositae Vol. I, Heywood, V.H., Harborne, J.B., Turner, B.L. Eds.; Academic Press, New York, 1977, pp 412-433.

35. Fukumaru, T.; Awata, H.; Hamma, N.; Komatsu, T. Agr. Biol. Chem. 1975, 39, 519-527.

36. Khafagy, S.M.; Metwally, A.M.; El-Ghazooly, M.G. Egypt. J. Pharm. Sci. 1982, 20, 115-120.

37. Saleh, M.A. Phytochemistry 1984, 23, 2497-2498.

38. Binder, R.G.; Chan, B.G.; Elliger, C.A. Agric. Biol. Chem. 1979, 43, 2467-2471.

39. Kobayashi, A.; Morimoto, S.; Shibata,Y.; Yamashita, K.; Numata, M. J. Chem. Ecol. 1980, 6, 119-131.

40. Campbell, G.; Lambert, J.D.H.; Arnason, J.T.; Towers, G.H.N. J. Chem. Ecol. 1982, 8, 961-972.

41. Stevens, K.L. J. Chem. Ecol. 1986, 12, 1205-1211.

42. Stevens, G.A.; Tang, C.S. J. Chem. Ecol. 1985, 11, 1411-1425.

43. Reisch, J.; Spitzner, W.; Schulte, K.E. Arzneim. Forsch. / Drug Res. 1967, 17, 816-840.

44. Towers, G.H.N. In Progress in Phytochemistry, Vol. 6. Reinhold, L., Harborne, J.B., Swain, T., Eds.; Pergamon Press, London, pp 183-202.
45. Wat, C.K.; Prasad, S.K.; Graham, E.A.; Partington, S.; Towers, G.H.N. Biochem. Syst. Ecol. 1981, 9, 59-62.
46. Arnason, J.T.; Philogène, B.J.R.; MacEachern, A.; Kaminski, J.; Leitch, L.C.; Morand, P.; Lam, J. Phytochemistry 1986, 25, 1609-1611.
47. McLachlan, D.; Arnason, T.; Lam, J. Biochem. Syst. Ecol. 1986, 14, 17-23.
48. Marchant. Y.Y.; Ganders, F.R.; Wat, C.K.; Towers, G.H.N. Biochem. Syst. Ecol. 1984, 12, 167- 178.
49. Marchant, Y.Y.; Towers, G.H.N. Biochem.. Syst. Ecol. 1986, in press.
50. Prévost, S.; Meier, J.; Chodkiewicz, W.; Cadiot, P.; Villemart, A. Mem. Soc. Chim. Paris 1961, 2171-2175.
51. Beny, J.P.; Dhawan, S.N.; Kagan. J; Sundlas, S. J. Org. Chem. 1982, 47, 2201-2204.
52. Kagan, J.; Arora, S.K. J. Org. Chem. 1983, 48, 4317-4320.
53. Fowlks, W.L.; Griffith, D.G.; Oginsky, E.L. Nature, 1958, 181, 571.
54. Daniels, F. J. Invest. Dermatol. 1965, 44, 259-263.
55. Ashwood-Smith, M. J.; Poulton, G.A.; Ceska, O.; Liu, M.; Furniss, E. Photochem. Photobiol. 1983, 38, 113-118.
56. Ashwood-Smith, M.J., Ceska, O.; Chaudhary, S.K.; Warrington, P.J.; Woodcock, P. J. Chem. Ecol. 1986, 12, 915-932.
57. Song, P.S.; Tapley, K.J., Jr. Photochem. Photobiol. 1979, 29, 1177-1197.
58. Bakker, J.; Gommers, F.J.; Nieuwenhuis, I.; Wynberg, H. J. Biol. Chem. 1979, 254, 1841-1844.
59. Yamamoto, E.; Wat, C.K.; MacRae, W.D.; Towers, G.H.N. FEBS Lett. 1979, 107, 134-136.
60. MacRae, W.D.; Irwin, D.A.J.; Bisalputra, T.; Towers, G.H.N. Photochem. Photobiophys. 1980, 1, 309-318..
61. Hudson, J.B.; Graham, E.A.; Towers, G.H.N. Photochem. Photobiol. 1982, 36, 181-185.
62. Arnason, J.T.; Wat, C.K.; Downum, K.; Yamamoto, E.; Graham, E.A.; Towers, G.H.N. Can. J. Microbiol. 1980, 26, 698-705.
63. Arnason, J.T.; Chan, G.F.Q.; Wat, C.K.; Downum, K.R.; Towers, G.H.N. Photochem. Photobiol. 1981, 33, 821-824.
64. Downum, K.R.; Hancock, R.E.W.; Towers, G.H.N. Photochem. Photobiol. 1982, 36, 517-532.
65. Downum, K.R.; Towers, G.H.N. J. Nat. Prod. 1983, 44, 98-103.
66. McLachlan, D.; Arnason, J.T.; Lam, J. Photochem. Photobiol. 1984, 39, 177-182.
67. Weir, D.; Scaiano, J.C.; Arnason, J.T.; Evans, C. Photochem. Photobiol. 1985, 42, 223-230.
68. McRae, D.G.; Yamamoto, E.; Towers, G.H.N. Biochim. Biophys. Acta 1985, 821, 488-496.

Chapter 18

Thiophenes and Acetylenes: Phototoxic Agents to Herbivorous and Blood-Feeding Insects

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Thiophenes and acetylenes of the plant family Asteraceae have been found to be highly phototoxic to insects. Effects on herbivorous insects include formation of necrotic lesions, growth reduction and mortality. Mosquito larvae can be controlled at concentrations as low as a few parts per billion. Efficient synthetic methods have been developed to produce naturally occurring compounds and derivatives for laboratory and field trials. A high temperature dilute acid technique has produced tritiated phototoxins for pharmacokinetic studies in insects. In fundamental studies on the photosensitization mechanism, laser flash photolysis has been used to determine triplet lifetimes of the sensitizer, rates of energy transfer to O_2 and electron transfer to acceptor, or from donor molecules. The available information suggests that this group of phototoxins has excellent potential for development as insecticides.

The discovery that many diverse secondary metabolites from different plant families are photosensitizers (1,2) suggests not only that phototoxicity has arisen independently many times in evolution as a defense mechanism but that it may have significant advantages in discouraging plant pests. Studies of these naturally occurring systems of defense provide, in addition, new models for the development of pest control agents. The present report concerns a large group of phototoxic compounds, the thiophenes and biosynthetically related polyacetylenes of the Asteraceae, their role as allelochemicals to insect pests, and their possible exploitation as insecticides. The light-mediated toxicity to organisms other than insects by substances from this plant family was established by Gommers' group in the Netherlands (3) and Towers' group in Canada (4). Subsequently, it was demonstrated that 9 out of 14 compounds tested were phototoxic to mosquito larvae at a concentration of 0.5 ppm under sources of

near UV irradiation (5). The activity was even greater in
sunlight and two compounds, alpha-terthienyl (α-T) (I)and a furano
acetylene (II) had exceptional insecticidal activity with LC_{50}'S
of 19 and 79 ppb respectively (6). This unusually high activity
led to the patenting of these substances as insecticidal agents
(7) and to the investigation of the potential of the compounds as
mosquito larvicides.

Herbivorous insects

While we may hypothesize that phototoxic chemicals in the
Asteraceae have arisen in the course of evolution through
selective pressures exerted by herbivorous insects or plant
pathogens, the study of photosensitization of insect herbivores
has been limited because of the large amount of the chemicals
required for careful investigation of the effect of these
substances on insect development. Downum et al (8) and Champagne
et al. (9) have demonstrated phototoxic effects in Manduca sexta
and Euxoa messoria induced by injested or topically applied α-T.
Later Champagne et al. (10) examined the effect of seven
acetylenes and thiophenes on three insect species. Several
(I,III,IV) but not all of these compounds were phototoxic to M.
sexta and/or E. messoria. However, Ostrinia nubilalis tended to
avoid the effects of photosensitization by burrowing into diet or
spinning silk, behaviors which may be adaptations to avoid
photosensitization by an insect which is known to feed on
phototoxic Asteraceae. Specifically these three studies indicated
that absorption of light by the phototoxic chemicals may induce
mortality, lengthen larval development time, and reduce feeding
and growth of sensitive insects. The most acute effects observed
were pupal deformities and necrotic lesions in the cuticle that
prevent ecdysis. The effects of photosensitization by acetylenes
and thiophenes of the Asteraceae on insect herbivores is
comparable to Berenbaum's description of the effects of
furanocoumarins of the Apiaceae on unadapted insects (11). It is
not surprising that both these families share an adapted insect
fauna that appears to tolerate or avoid the photosensitizing
chemicals.
        Phototoxic acetylenes and thiophenes presumably provide
enhanced protection of the plant by virtue of their involvement in
high energy photochemical processes and the catalytic nature of
singlet oxygen generation which they mediate.
        Thus, plants may minimize their metabolic investment in
chemical defenses by a relatively free commodity, light, which is
always available in suitable plant habitats. These compounds are
found in a variety of above ground locations. For example,
phenylheptatriyne (PHT) (III) is found in high concentrations in
the cuticle of leaves of Bidens pilosa (1), while thiophenes are
found in high concentrations in prominent marginal leaf glands of
Porophyllum spp (Arnason, unpublished). Many members of the
subtribe Pectinidae are rich in thiophenes in their above ground
parts (38).
        It should be emphasized, however, that without
photosensitizing radiation, acetylenes and thiophenes still
possess many of the insect deterrent effects observed with other

non-photosensitizing plant secondary metabolites e.g. feeding
deterrence, growth reduction, and reduced nutrient utilization
(8,9,10,12). The presence of the compounds in moderately high
concentrations in the roots of many Asteraceae (1) still suggests
a defensive role, although more expensive metabolically.
Differentiation between light and dark toxicity for herbivorous
insects is not as marked in microorganisms or mosquito larvae,
possibly because of the lack of light penetration into larger
organisms. Other reports of the insecticidal activity of
acetylenes and thiophenes exist in the literature (e.g. 12, 14,
and 15), but little attention was placed on the possible role of
photosensitization in these investigations.
      Recent studies have attempted to explain why some insect
species are more sensitive to α-T than others. For example, late
instar M. sexta and Pieris rapae are very sensitive to α-T but O.
nubilalis and Heliothis virescens are more tolerant (Table 1).

Table 1

Contact Phototoxicity of α-terthienyl to last instar lavae

	LC_{50} ($\mu g/g$)
Manduca sexta	10
Pieris rapae	15
Heliothis virescens	474
Ostrinia nubilalis	698

Note: Larvae in their last instar were weighed and treated with
α-terthienyl dissolved in acetone at the following rates of
application 0, 1, 3, 30, 100, 300, 1000, $\mu g/g$ larvae. Insects
were irradiated under blacklight blue lamps for 12 hours at
$2w/m^2$ and LC_{50} values calculated from a probit analysis of the
mortality data.

      In order to explain these differences, tritiated phototoxins
were produced in high yield (80-100%) by a high temperature dilute
acid technique developed by Werstiuk (16) for metabolic studies.
The procedure was optimized by preliminary studies using D_2O as
the isotope source before final incorporation of tritium from HTO.
The nature of the electrophilic exchange of aromatic protons
permits prediction of the relative rates of exchange which has
been confirmed by NMR spectroscopy. Both ^3H α-T and ^3H-Me-α-T
have been produced by this procedure.
      Pharmacokinetic studies (17) have shown that, after a topical
application of the label to the test insects, the half time for
clearance was very slow for sensitive M. sexta (48 hr), but was
much more rapid for tolerant O. nubilalis (6 hr) and H. virescens
(20 hr). In addition, α-T administered in the diet was found to
cross the gut and enter the hemolymph to a greater extent in M.
sexta than in the two resistant species. At least one major

metabolite has been detected in feces of these insects but its
chemical identity is not yet known. Apparently, efficient
metabolism and clearance of α-T is one mechanism by which some
herbivorous insects can deal with this phototoxic allelochemical.

Biological effects at the third trophic level have also been
demonstrated with α-T. Incorporation of 100 µg/g of α-T into
diets of O. nubilalis significantly reduced the rate of
parasitization, survival to pupation and adult emergence of the
hymenopterous parasitoid, Diadegma terebrans relative to controls
(18). Detection of α-T in the adult parasitoid suggested that
photosensitization of the parasitoid may be possible although it
has not yet been directly demonstrated.

Mosquito larvae

The evaluation of α-T or other molecules as commercial larvicides
has been hampered by lack of an efficient synthesis. Existing
syntheses were either highly inefficient or were multistep
procedures. Application of a catalyzed coupling reaction, first
reported by Tomao et al. (19) on a millimole scale, has made it
possible to produce α-T on 50 g scale using readily available
2-bromothiophene and 2,5-dibromothiophene as starting materials.
The process is a one pot Grignard-Wurtz reaction involving a Ni
catalyst which drastically reduces the formation of byproducts,
followed by a simple purification step to give 99% pure material
(20). A recent improvement is the elimination of diethyl ether,
an industrially undesirable solvent, for which a process patent
application (21) has been filed.

Field trials have been undertaken at a deciduous forest site
for 3 years and at a boreal site for 2 years. Spray application
tests of α-T formulated in ethanol to natural snow melt pools that
are Aedes spp. breeding sites, revealed that effective control
could be achieved at an application rate as low as 10 g active
ingredient/ha within 7 days post application (22). More rapid
(1-2 days post application) and reliable control is achieved at
100g a.i./ha. This is comparable to the efficacy of currently
used organophosphates (e.g. Temephos and Pirimiphos Methyl) but
less than that of some pyrethroids. Recent work has focussed on
the development of a suitable concentrate and an oil-based
spreading formulation that are convenient to use and are as
effective or better than the original ethanol formulation.

A potential limitation for phototoxic control agents is
diminished light. We have observed reduced efficacy in turbid
pools as compared to clear pools (Table 2) or under heavy overcast
as compared to clear, sunny conditions. However, within a short
time, there is a comparable toxicity at the reduced light level
suggesting that the light requirement is saturated fairly
rapidly.

Evaluation of environmental safety of α-T with respect to non-target organisms requires more precise toxicity data. This has been achieved by laboratory trials or simulated pond trials at the field site with field collected mosquitos and non-target organisms held in bioassay cages and placed in plastic wading pools filled with pond water. Target and non target data from this trial are shown in Table 3. Application of 0.10 kg/ha of α-T can effectively control mosquito larvae with minimal effects toward damsel and caddis fly larvae, trout, or snails. These non-target results with damsel and caddis fly are better than currently used pyrethroids. However Daphnia and midge are adversely affected at this rate of application by α-T.

Table 2

Larvicidal Efficacy of α-T as a function of light and time

Treatment	Days post-application	EC_{90}
		(g/ha)
Turbid pool*	1	4365
	2	132
Clear pool	1	104
	2	60

* Fine sediment was disturbed in pools to create a situation where light penetration of water was reduced. EC_{90} for mosquito larvicide activity was determined as described in (20).

Table 3

Target and Non-Target Data for Alpha Terthienyl

	EC_{50} (kg/ha)	LC_{50} (ppb)	reference
Target organism			
mosquito larvae	0.046	15–30	6,22
Non-target organisms			
damsel fly larvae	0.38	-----	22
caddis fly larvae	1.32	-----	22
midge larvae	0.178	-----	22
daphnia larvae	0.044	-----	22
trout fingerlings	10	-----	22
snail	10	-----	22
tadpole	-----	18–111	23
fathead minnows	-----	50–650	24

Note EC_{50} values are based on surface area applications and LC_{50} values are calculated on a concentration basis.

Kagan (23-24 and this volume) has raised environmental concerns with respect to use of α-T as a larvicide because of its toxicity to tadpoles and fathead minnows when formulated in dimethyl sulfoxide (DMSO). While the LC_{50} values for these organisms are higher than for mosquito larvae (Table 3) and the use of DMSO as a formulating agent may be questioned, there is clearly a need for more non-target data.

Evaluations of the toxicology of α-T show that it is non-toxic to mice at 300 mg/kg by oral or dermal routes of administration several days after administration (20), but that it is toxic to rats at 1000 mg/kg in 24 hr. Physiological effects at high doses include CNS depression and hypotensive activities [e.g. decreased motor activity and loss of righting reflex (25)]. Acetylenes and thiophenes are non-mutagenic (26), but can photosensitize human skin at high doses (1). The latter was not found to be a problem under dilutions used for larviciding.

Analogues and derivatives of α-T

A number of investigator have synthesized α-T derivatives and analogues, usually by a multistep procedure involving various cyclization reactions (see ref 20). Several 1,3-butadiene and thiophene derivatives prepared in one of these procedures have been reported to be phototoxic to mosquito larvae on a qualitative basis (27). Recently, the catalyzed Grignard coupling reaction was used to produce a series of compounds (28) structurally related to α-T. LC_{50} values between 15 and 1000 ppb were reported for these compounds. Only one compound, a monomethyl derivative of α-T (V), was more toxic than the parent compound (I).

Considerable success in the pesticide industry has been achieved by the production of designed analogues of natural products such as pyrethrins and juvenile hormones. We have investigated the analogues and derivatives of α-T in relation to their photochemical and physical properties (29). Laser flash photolysis has demonstrated that most of the compounds have long
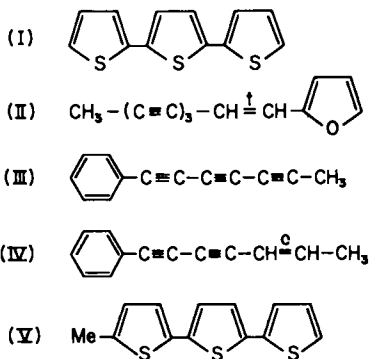
Structures I-V

triplet lifetimes, and that these triplets are efficiently
quenched by O_2 to produce singlet O_2 with a quantum yield between
0.4 and 1.0; the quantum yield is 0.86 for α-T itself which is
one of the most efficient singlet O_2 generators in the series
tested. Octanol-water partition coeficents (P) have been
estimated to range from log P values of 4 to 7. Multiple
regression of the data suggested that toxicity was at least
partially predictable on the basis of photochemistry and partition
coefficients. A design model based on this and other information
should lead to the production of more efficient phototoxins in the
future.

Mechanisms of Action of α-T, its synthetic analogues and derivatives, and naturally occuring acetylenes

Assignement of mechanisms of action for these phototoxins is
controversial. At the molecular level, α-T was originally
reported to have no O_2 requirement for phototoxic action (30).
Other reports have now clearly established singlet O_2 generation
as the in vivo and in vitro mode of action of α-T (1,2).
Subsequently, conflicting interpretations have emerged suggesting
that polyacetylenes such as phenylheptatriyne (PHT) might act by a
wholly photodynamic mechanism (31) or by competing photodynamic
and free radical mechanisms (32).

The recent application of laser flash photolysis has resolved
some of the mechanistic controversies and provides new insight
into the photochemistry of these molecules (33-35). With both
thiophenes and acetylenes, laser excitation results in the
formation of strong triplet signals with long lifetimes (28 μs for
PHT in MeOH, 30 or 57 μs for α-T in MeOH or EtOH respectively).
The triplets are efficiently quenched by O_2, and by methyl
viologen but not by amines. With PHT, quenching by O_2 and
electron transfer to methyl viologen occur with comparable rate
constants (34), but the back reaction of the electron transfer
(presumably from PHT$^+$ to methyl viologen) was less efficient
(50-60%) (35). Recently, detection of a free radical signal by
ESR spectroscopy, in liposomes containing PHT, has been achieved
after irradiation with UV-A (36). Thus direct and indirect
evidence suggests that PHT may act by generation of free radicals
or singlet oxygen.

At the biochemical level, Kagan (27) has reported
inactivation of the acetylcholinesterase of mosquito larvae under
conditions that induce severe insect mortality. A more immediate
effect may be the destruction of anal gill membranes of larvae
which can be directly observed by light microscopy.
Loss of membrane integrity results in the release of electrolytes
into water, and this is greatly enhanced under photosensitizing
conditions (Table 4) at sublethal conditions. With herbivorous
insects, there is evidence of interference with melanization and
scleratization of pupae (8) and damage to the gut (10) of larvae,
in addition to the dramatic cuticular lesions.

Table 4

Halide detected in demineralized Water after 2 hours
(meq/L. ±S.D.)

	−αT	+αT
−UV	0.080 ±0.012	0.075 ±0.007
+UV	0.105 ±0.004	0.237 ±0.0002

Eighty Aedes atropalpus fourth instar larvae were treated with
combinations of 100 ppb α-T and near UV $(4w/m^2)$ in 8 ml
demineralized water. Halide leakage was detected with a Buchler
Chloridometer. No mortality was observed in the 2 h. treatment.

Conclusion

Resistance and cross-resistance are major problems with currently
used insecticides (37). The development of new pest control
agents is, for this reason, essential. With α-T, cross resistance
to organophosphates does not appear to be a problem (24).
Resistance to phototoxins may be slow to develop in aquatic larvae
because of the rapid and novel mode of action of these substracts.
Final commercial development of thiophenes or acetylenes will
depend, however, on many factors including proprietary rights,
market size, and suitability for registration. Their demonstrated
mode of action and efficacy and the preliminary data on toxicology
and non-target effects suggests that these substances have an
excellent potential for taking their place alongside of other
phototoxic insecticides, herbicides and chemotherapeutic agents
(1,2) in the new phototoxin technology that is developing in
industry.

Acknowledgment

Literature Cited

1. Towers, G.H.N. Can. J. Bot. 1984 62 2900-2911
2. Knox, J.P.; Dodge, A.D. Phytochem. 1985, 24 889-896
3. Gommers F.J. Nematology 1972, 18 458
4. Camm, E.L.; Towers G.H.N.; Mitchell, J.C., Phytochem 1975,
    14,2007-2011

5. Wat C.K.; Prasad, S.K.; Graham, E.A.; Partington, S.; Arnason, T., Towers, G.H.N.; Lam, J. Biochem. Syst & Ecol. 1981, 9, 59-62

6. Arnason, T.; Swain, T.; Wat, C.K.; Graham, E.A.; Partington, S.; Towers, G.H.N. and Lam J. Biochem. Syst. & Ecol. 1981, 9, 63-68

7. Towers, G.H.N.; Arnason J.T.; Lambert J.D.H.; Wat C.K., Can Pat. 1,173,743 1984

8. Downum K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest. Biochem Physiol. 1984 22 104-109

9. Champagne D.E.; Arnason J.T.; Philogene B.J.R.; Campbell, G.; and McLachlan, D. Experienta 1984, 40 577-578

10. Champagne, D.E., Arnason J.T., Philogène, B.J.R.; Morand P.; Lam J. J. Chem. Ecol. 1986, 12 835-858

11. Berenbaum, M. Evolution 1983, 37 163

12. McLachlan, D.; Arnason, J.T.; Philogène, B.J.R.; Champagne, D. Experienta 1982, 38 1061-1062

13. Morallo-Rejesus, B.; Decena, A. Philipp. J. Crop Sci. 1982, 7 31-36

14. Kakajima, S; Kawazu K. Agric. Biol. Chem. 1977, 41 1801-4

15. Kawazu, K.; Ariwa, M.; Kii, Y. Agric. Biol. Chem. 1977, 41 223-224

16. Werstiuk, N.H.; Kadai, T. Can J. Chem. 1973, 51 1485

17. Iyengar, S.; Arnason T.; Philogène, B.J.R.; Werstiuk, N.; Morand P., Abstr. Proc. I.U.P.A.C. Sixth Internat'l Pesticide. Conf. 1986, Ottawa

18. MacDougall, C. MSc. thesis., University of Ottawa, Ottawa, 1986

19. Tomao, K.; Kodoma, S.; Nikajima I; Kumada, M.; Mirato, A.; Suzuki, K. Tetrahed. Lett. 1983, 38; 3347-3354

20. Philogène, B.J.R.; Arnason J.T., Berg C.W., Duval F.; Champagne D.; Taylor R.G.; Leitch, L.C.; Morand, P. J.Econ. Ent. 1985, 78 121-126

21. Morand, P.; MacEachern A.; Leitch, L.C.; Arnason, J.T. Can. Patent application filed July, 1986

22. Philogène, B.J.R.; Arnason, J.T.; Berg C.W.; Duval, F.; Morand, P. J. Chem Ecol. 1986, 12 893-898

23. Kagan, J.; Kagan, P.A.; Buhse, H.E. J. Chem Ecol. 1984, 10 1115-1122

24. Kagan, J.; Kagan E.D.; Seigneurie, W. Chemosphere 1986, 15, 49-57

25. Towers, G.H.N.; Duangto, K.; Arnason J.T. 1986, unpublished results

26. MacRae, W.D., Chan G.F.Q.; Wat C.K.; Towers, G.H.N.; Lam, J. Experientia 1980, 36 1096-1097

27. Kagan, J.; Beny, J.P.; Chan G.; Dahwan, S.N.; Jaworski J.A.; Kagan, E.D.; Kassner, P.D.; Murphy, M.; Rogers, J.A. Insect Sci. Appl. 1983, 4 377-381

28. Arnason, J.T.; Philogène B.J.R.; Berg, C.; Mac Eachern, A.; Kaminski, J.; Leitch, L.C.; Morand, P.; Lam, J. Phytochem. 1986, 25 1609-1611

29. MacEachern, A.; Scaiano, J.C.; Morand, P.; Arnason, J.T., Campos, F.; Philogène, B.J.R. Abst. Proc. I.U.P.A.C. Sixth Internat'l Pesticide Conf., Ottawa, 1986

30. Kagan, J.; Gabriel, J.R.; Reed, S.A. Photochem. Photobiol.
    1980, 31 465-469
31. Kagan, J.; Tadena Wielant, K.; Chan, G.; Shawan, S.N.;
    Jaworsky, J. Photochem. Photobiol. 1984, 39 465-467
32. Evans, C; Weir, D.; Scaiano, J.C.; MacEachern, A.; Arnason,
    J.T.; Morand, P.; Hollebone, B.; Leitch, L.C.; Philogène
    B.J.R. Photochem. Photobiol. 1986, in press.
33. Weir, D.; Scaiano, J.C.; Arnason, J.T.; Evans, C. Photochem.
    Photobiol. 1985, 42 223-230
34. Reyftmann, J.P.; Kagan J.; Santos, R.; Morlierre, P.
    Photochem. Photobiol. 1985, 41 1-7
35. MacRae, D.; Yammamoto, E.; Towers, G.H.N. 1986. Photochem.
    Photobioll. in press.
36. Kagan, J.; Hasson, M.; Grynspan F. Biochem. Biophys Acta
    1984, 802 442-447
37. Dover, M.J.; Croft, B.A. Bioscience 1986, 36 78-85
38. Downum, K.R.; Keil, D.J.; Rodriguez, E. Biochem. Syst. and
    Ecol. 1986, 13, 109-113.

Chapter 19

Photodynamic Action of Hypericin

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Hypericin, a photodynamic quinone, occurs in
certain species of the genus Hypericum (the St.
John's worts). Aspects of its photobiology and
photochemistry, especially in relation to its
ability to generate singlet molecular oxygen, have
been investigated. Its phototoxicity, including
that towards Manduca sexta larvae, is also
discussed.

The quinones provide many examples of naturally occuring
photodynamic compounds, and hypericin, found predominantly in the
Hypericaceae is historically the most important of these (1,2).
The photosensitization of grazing animals following the ingestion
of certain Hypericum species (the St. John's worts) is due to the
presence of hypericin (3,4). This condition, hypericism,
manifest in skin irritation and inflammation, is most commonly
caused by the ingestion of Hypericum perforatum and has been most
prevalent in North America and Australia (1,5).

Chemistry

The structure of hypericin and its biosynthetic pathway were
elucidated by Brockmann (7,8). Hypericin (4,5,7,4',5',7'-
hexahydroxy-2,2'-dimethylnaphthodianthrone) is a highly condensed
quinone and often occurs in the presence of closely related
photodynamic compounds, most commonly pseudohypericin (9).

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Hypericin

$R^1=R^2=CH_3$

Pseudohypericin

$R^1=CH_2OH$ $R^2=CH_3$
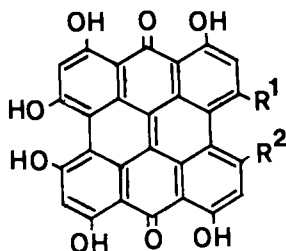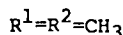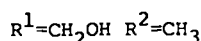
In ethanol, hypericin and pseudohypericin display identical and distinctive absorption spectra, with a series of absorption maxima between 500 and 600nm, and marked red fluorescence (10,11). They can be distinguished by their spectra in acidic ethanol and aqueous alkali (10). Hypericin is photostable in both aqueous and organic solvents.

Distribution

The largest survey of the distribution of hypericin within the Hypericaceae revealed that approx. 60% of the 200 species investigated contained hypericins. These species were concentrated in the sections Euhypericum and Campylosporus (12). This study utilised a leaf print technique that was unable to discriminate between hypericin and pseudohypericin. These compounds do differ in their distribution between species. H. perforatum contains both, H. hirsutum only hypericin and H. montanum and H. crispum only pseudohypericin (8). Their distribution within plant tissue also differs widely among species. In H. perforatum the leaves, stem and flowers contain the hypericins, whereas in H. hirsutum hypericin occurs only in the multicellular trichomes of the calyx (10). In all cases the hypericins are restricted to discrete glands.

Interestingly, the hypericin molecule appears to have other diverse occurrences in nature. The most notable examples are as the chromophore of the photoreceptor of Stentor coeruleus (a blue-green ciliate) (13) and in the integument of an Australian insect (Nipaecoccus aurilanatus) (14). In addition, buckwheat (Fagopyrum esculentum) contains fagopyrin, a derivative of hypericin (9), the mould Penicilliopsis clavariaeformis contains pencilliopsin which can be oxidised and irradiated to form hypericin (9) and the ciliate Blepharisma contains a pigment which is a possible polymer of hypericin (5).

Hypericin and singlet molecular oxygen

Early studies on the effect of hypericin on mammals demonstrated that its photosensitizing action required visible light and oxygen, i.e. was photodynamic. The production of singlet molecular oxygen by certain photodynamic reactions and its role as the toxic species in photooxidative damage has since been demonstrated (15). We have recently isolated hypericin from H. hirsutum and investigated its potential to photogenerate singlet oxygen (10).

Purified hypericin was observed to promote oxygen consumption from aqueous solutions when irradiated in the presence of imidazole (capable of reacting with singlet oxygen). This photooxidation was promoted in the presence of deuterium oxide and diminished by the addition of azide ions, suggestive of singlet oxygen involvement. In a further model system, the irradiation of hypericin was observed to promote the peroxidation of methyl linolenate, as measured by the appearance of ethane and malondialdehyde. Linolenate oxidation was reduced when crocin (a water soluble carotenoid capable of the efficient quenching of singlet oxygen) was added to the reaction mixture. In this system a concentration of hypericin greater than 10uM was observed to reduce lipid peroxidation relative to controls without hypericin (unpublished observation). This may reflect the direct scavenging of lipid radicals by hypericin. In both of the above systems the use of filters indicated that the effective irradiation was 500-600nm.

These observations clearly demonstrate the ability of hypericin to promote type II photodynamic reactions. Hypericin is thus potentially disruptive of biological systems in which it is irradiated in proximity to vulnerable cellular components such as the unsaturated lipids of membranes (2,15). In addition, evidence for the photogeneration of superoxide anions by the irradiation of hypericin in a reducing environment (in the presence of methionine) has been obtained in a system involving the reduction of nitroblue tetrazolium (unpublished observations). The extent to which type I photodynamic reactions (including the generation of superoxide anions) are a component of the photodynamic damage sensitized by hypericin is unknown.

Phototoxic action of hypericin

Photodynamic reactions are generally not species specific. Although hypericin potentially has a wide toxicity, its action will be greatly modulated by variations in its sequestration and metabolism among species and within tissues. As yet the phototoxicity of hypericin has been investigated in only a few systems.

As already stated the early investigations upon the toxicity of hypericin were conducted due to the prevalence of hypericism (5). Hypericin must be ingested by mammals to result in hypericism and, unlike the furanocoumarins, does not appear to be

absorbed through the outer layers of the epidermis (5). After ingestion animals remain sensitive to sunlight for a week or more. Skin irritation and inflammation is most severe in regions of unpigmented skin devoid of hair i.e. the mouth, nose and ears (5).

As already pointed out hypericin occurs as a component of the photoreceptor of S. coeruleus and predisposes this organism to lethal photodynamic injury (16). Exogenous hypericin promotes this injury and the use of quenchers in this system provides evidence of the involvement of singlet oxygen.

The precise role of hypericin in Hypericum species is unclear, although it would appear to be a defensive one. Although this compound can be a source of irritation to mammals, it is rarely fatal and does not appear to deter grazing animals (5). It has been suggested that hypericin may act as a deterrent to phytophagous insects (5), a frequently proposed role for photosensitizing plant secondary metabolites (2,17). Hypericin is reported to be phototoxic to mosquito (Aedes atropalpus) larvae (17).

We have utilised third instar larvae of the tobacco hawkmoth (Manduca sexta, Lepidoptera: Sphingidae) as a model insect herbivore for the investigation of the phototoxicity of hypericin towards insects. The normal host range of M. sexta does not include any species of the Hypericaceae.

Hypericin, isolated as described previously (10), was observed to be phototoxic to M. sexta larvae. At the moderate radiance level used in this study (22 Wm^{-2}, provided by white fluorescent tubes) the LD_{50} was found to be 16µg/g larval initial fr.wt., which represents approx. a 1µg dose to a third instar larva (Table I). In these experiments, the hypericin was administered to larvae on tobacco leaf discs (7mm diameter) after 1h of starvation from an artificial diet, and observations were made during the subsequent continuous irradiation for up to 48h. No mortality or any effects upon weight gain were observed in the hypericin treated but dark-maintained control larvae.

Reduced irradiance resulted in decreased mortality, although after 48h the surviving larvae at the lower light levels displayed reduced weight gain relative to dark controls. The modulation of light quality by a cut off filter (allowing irradiation only with wavelengths greater than 500nm) reduced the mortality rate by only 20%, confirming that active wavelengths in hypericin toxicity are greater than 500nm (data not shown).

If after consumption of the hypericin treated leaf discs the larvae were maintained in darkness on an artificial diet, the phototoxic effect upon subsequent irradiation was rapidly lost. Mortality was reduced to 6% if irradiation was delayed for 8h after treatment (Table I). If the larvae were not supplied with artificial diet during this period of darkness, the potential for a high mortality rate upon subsequent irradiation of the larvae supplied with diet was retained. These observations suggest that hypericin may not be readily absorbed by the gut but photoactive at the gut wall and rapidly lost from the gut by excretion.

Table I. Lethal phototoxicity of hypericin towards <u>Manduca sexta</u>
larvae. Hypericin administered on tobacco leaf discs.
Mortality monitored after 48h of irradiation.

hypericin dose (μg/larva)[a]	irradiation conditions (Wm^{-2})	time in darkness between treatment and irradiation (h)	percentage mortality[b]
0	22	0	0
0.1	"	"	0
0.3	"	"	6
0.6	"	"	12
1.0	"	"	47
1.5	"	"	89
2.5	"	"	100
2.5	DARK	"	0
2.5	10	"	15
2.5	4	"	0
2.0	22	0	81
2.0	"	2	34
2.0	"	4	11
2.0	"	8	6

a. average initial fresh weight (3rd instar) = approx. 60mg.
b. at least 15 insects per treatment.

Conclusion

These preliminary observations demonstrate that orally administered hypericin is toxic to larvae of <u>M. sexta</u>, a herbivore unaccustomed to hypericin containing plants. This toxicity has an absolute light dependence at the dose levels used in this study, with no mortality or growth retardation observed in dark maintained controls. In this case a maximum radiance of 22 Wm^{-2} was used, considerably less than daylight. The LD_{50} could therefore be reduced in a natural environment. In this study the hypericin equivalent to that contained in approx. 50 glands of <u>H. hirsutum</u> (10) was lethal to a third instar larva. The leaf tissue of <u>H. perforatum</u> contains hypericin at levels up to 1mg/g dr.wt. (12). Visible irradiation (500-600nm) is required for hypericin toxicity contrasting with that of other plant metabolites capable of photosensitizing <u>M. sexta</u> larvae. A thiophene, ∝-terthienyl, required UV irradiation (320-400nm) for its action (18).
    The possibility that hypericin acts as a deterrent to phytophagous insects requires further toxicity tests and a survey of insects that utilise <u>Hypericum</u> species. A beetle, <u>Chrysolina</u>

brunsvicensis, does feed upon H. hirsutum, using hypericin as a
feeding cue (19). Other Chrysolina species have been used
successfully as a means of biological control of H. perforatum in
Australia (5, 20).
     An area of ignorance highlighted by this possible case of
coevolution, is the means by which organisms are able to tolerate
photodynamic action. Photodynamic damage may be reduced by
behavioural or physiological mechanisms. The mechanisms whereby
biological systems could prevent the generation of singlet
molecular oxygen or withstand its specific but disruptive
oxidations would be of especial interest.

Literature cited

1. Blum, H.F. 'Photodynamic action and diseases caused by
    light'; Reinhold Publishing Company, New York, 1941.
2. Knox, J.P.; Dodge, A.D. Phytochem. 1985, 24, 889-896.
3. Horsley, C.H. J. Pharmcol. 1934, 50, 310-322.
4. Pace, N. Amer. J. Physiol. 1942, 136, 650-656.
5. Giese, A.C. Photochem. Photobiol. Rev. 1980, 5, 229-255.
6. Towers, G.H.N. Prog. Phytochem. 1980, 6, 183-202.
7. Brockmann, H.H. Prog. Organic Chem. 1952, 1, 64-82.
8. Brockmann, H.H. Proc. Chem. Soc., London 1957, p. 304.
9. Thompson, R.H. 'Naturally occurring Quinones';
    Academic:London, 1971.
10. Knox, J.P.;Dodge, A.D. Plant Cell Environ. 1985, 8, 19-
    25.
11. Scheibe, G.; Schöntage, A. Chem. Ber. 1942, 75, 2019-
    2026.
12. Mathis, C.; Ourisson, G. Phytochem. 1963, 2, 157-171.
13. Walker, E.B.; Lee, T.Y.; Song, P.S. Biochim. Biophys.
    Acta. 1979, 587, 129-144.
14. Cameron, D.W.; Raverty, W.D. Aust. J. Chem. 1976, 29,
    1523-1533.
15. Foote, C.S. In 'Free Radicals in Biology'; Pryor, W.A.
    Ed; Academic:London, 1976; Vol. II, p.85.
16. Yang, K.C.; Prusti, R.K.; Walker, E.B.; Song, P.S.; Watanabe,
    M.; Furuya, M. Photochem. Photobiol. 1986, 43, 305-310.
17. Arnason, T.; Towers, G.H.N., Philogene, B.J.R.; Lambert,
    J.D.H. In 'Plant Resistance to Insects'; Hedin, P.A.
    Ed; ACS Symposium Series No. 208, American Chemical
    Society: Washington, D.C., 1983, pp. 139-51.
18. Downum, K.R.; Rosenthal, G.A.; Towers, G.H.N. Pest.
    Biochem. Physiol. 1984, 22, 104-109.
19. Rees, C.J.C. Entomol. Exp. Appl. 1969, 12, 565-583.
20. Clare, N.T. 'Photosensitization in diseases of domestic
    animals' Commonwealth Agric. Bureaux; England, 1952, pp.
    14-15.

Chapter 20

The Fungal Photosensitizer Cercosporin and Its Role in Plant Disease

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Cercosporin is a photosensitizing toxin produced by fungal plant pathogens in the genus Cercospora. Cercosporin produces singlet oxygen and superoxide when irradiated by light, and damages plants by causing a peroxidation of the membrane lipids. This membrane damage leads to a loss in membrane fluidity, leakage of nutrients, and rapid death of the plant cell. Cercosporin is toxic to mice and bacteria in addition to plants, and attempts to select plant cells in vitro for resistance to cercosporin have not been successful. A large number of fungal species, however, are resistant to cercosporin. Carotenoids and the fungal cell wall appear to play a critical role in the resistance of fungi to cercosporin.

Cercosporin (1) is a toxin which appears to play an important role in plant diseases caused by members of the fungal genus Cercospora. Cercospora species incite diseases on a large number of host species worldwide, including such crops as corn, soybean, sugar beet, peanut, banana, and coffee. Losses from these diseases can be devastating. In 1985 in North Carolina alone, Cercospora leaf spot of peanuts caused an estimated 5 million dollar loss with an additional 13 million dollars spent on control measures to combat the disease; these costs represented almost 15% of the total crop value (1).

Cercospora species are aerial pathogens. Spores produced by these organisms germinate on the leaf surface and enter the leaf through the stomata. Fungal mycelium then ramifies through the leaf intercellular spaces, killing the cells and causing severe blighting of the leaf tissue. For many years, it had been observed that high light intensities were required for symptom development on infected plants (2-4). This effect was so striking that it actually led to the recommendation in the 1940's that bananas be grown under partial shade to control the disease (5). Although these observations suggested that some type of light-activated compound was involved in
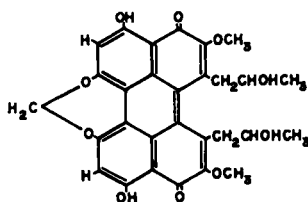
1   Cercosporin

disease development, it was not until the 1970's that cercosporin
was identified and shown to be light activated (6-8).

Cercosporin

Cercosporin was first isolated in 1953 by Deutschmann from
Cercospora kikuchii, a soybean pathogen (9). In 1957 Kuyama and
Tamura independently isolated the compound from the same fungus, and
named it cercosporin (10). Cercosporin has since been isolated from
a large number of Cercospora species (11-16) and Cercospora-infected
plants (10,13,16). Its characterization and structure were reported
independently by Lousberg and co-workers (6) and Yamazaki and Ogawa
(7). Further studies of its stereochemistry have been reported by
Nasini and co-workers (17). Okubo et al. showed that cercosporin is
biosynthesized in the fungus by the polymerization of acetate and
malonate via the polyketide pathway (18). Nutritional and
environmental conditions regulating toxin biosynthesis by the fungus
have also been reported (19).
        Cercosporin's photosensitizing activity was first demonstrated
by Yamazaki and co-workers in 1975 (8). They showed that
cercosporin was toxic to mice and bacteria only when they were
exposed to light. They further demonstrated an oxygen requirement
by the photooxygenation of dimethylfuran, and showed that
cercosporin was capable of degrading amino acids.
        The first report of the photoactivated toxicity of cercosporin
to plants was that of Macri and Vianello (20). They demonstrated
that cercosporin caused ion leakage from corn, potato, and beet
tissues only when they were irradiated by light. This effect was
observable within 15-30 minutes after treatment and required oxygen.
They further found that several synthetic antioxidants could
partially inhibit the cercosporin-induced ion leakage.
        Our studies on the photosensitizing activity of cercosporin
have been done with plant suspension cultures, single-celled
cultures of undifferentiated heterotrophic callus cells grown in
liquid medium (21). These cultures are ideal for such studies
because they grow equally well in the light and dark and lack
photosynthetic pigments which could interfere with light absorption.
The use of single cell cultures also overcomes other problems
encountered with whole tissue studies, because the cells can be
exposed uniformly to the toxin, and toxin effects can be quantified
by counting the number of cells killed. The suspension cultures were
found to be very sensitive to cercosporin. For example, at 5 μM

cercosporin, all cells in a 50 ml tobacco or sugar beet suspension culture (approximately 10^6 cells) were killed within 4 hours when irradiated with fluorescent lights at an intensity of 20 joules· m^{-2}·sec^{-1} (21). At lower cercosporin concentrations, longer incubation times were required, but even at 0.2 μM, all cells were dead within 48 hours. By contrast, in the dark no cell death occurred even at cercosporin concentrations of up to 40 M for up to 7 days. As expected, killing of cells by cercosporin was directly proportional to light dose, with increasing intensity compensating for decreasing exposure times. The wavelength of light was also critical; an action spectrum of the killing of cells by cercosporin was found to be in close agreement with the absorption spectrum of cercosporin (21).

Cercosporin appears to be able to generate both singlet oxygen and superoxide when irradiated with light in vitro (22). Cercosporin, in the presence of light, oxygen, and the reducing substrate methionine was able to reduce p-nitro blue tetrazolium (a dye readily reduced by superoxide). Superoxide dismutase inhibited this reaction, whereas the singlet oxygen quencher Dabco (1,4-Diazabicyclo octane) had no effect. Cerosporin also reacted with cholesterol in the presence of light and oxygen to generate the 5α-hydroperoxide of cholesterol, demonstrating the production of singlet oxygen. Dobrowolski and Foote recently determined the quantum yield of singlet oxygen formation sensitized by cercosporin to be 0.81 ± 0.07 (23).

These results do not prove that both singlet oxygen and superoxide play a role in the killing of cells by cercosporin, but several lines of evidence suggest that both may be involved. The killing of suspension culture cells by cercosporin could be significantly inhibited by the addition of two singlet oxygen quenchers to the cell culture medium, Dabco and bixin (21) (bixin is a carotenoid carboxylic acid which has the same isoprenoid chain length as β-carotene, but is somewhat soluble in aqueous solutions). In addition, a low level of resistance to cercosporin was expressed by a tobacco cell culture mutant, selected for resistance to paraquat, which has elevated levels of superoxide dismutase activity (24). Plants regenerated from this mutant showed no symptoms when sprayed with a cercosporin solution and showed less ion leakage following cercosporin treatment than normal tobacco tissue (Tanaka, K., Kyoto Prefectural University, personal communication, 1986).

Toxicity to Plant Cells

The most pronounced effect seen in cercosporin-treated plant tissues is damage to cellular membranes. Studies on the ultrastructure of Cercospora leaf blight of sugar beets (25) and of cercosporin-treated sugar beet leaves (26) showed membrane damage at early stages after infection or toxin treatment. Cercosporin also caused bursting of plant protoplasts (27) and leakage of ions and of the vacuolar pigment betalain (20,27) from treated cells. These effects were very rapid, suggesting that cercosporin has a direct effect on membranes. Changes in electrolyte leakage from tobacco and sugar

beet leaf disks, for example, could be detected within 1-2 minutes after treatment with cercosporin in the light (27).

Evidence from several laboratories demonstrates that this membrane damage is due to peroxidation of the membrane lipids by cercosporin. Cavallini and co-workers (28) demonstrated peroxidation of cellular constituents in vitro. They observed the formation of malondialdehyde (a breakdown product of lipid hydroperoxides) and O_2 consumption by liposomes and pea and rat liver mitochondria treated with cercosporin. These reactions could be inhibited by the singlet oxygen quenchers dimethylfuran and β-carotene, and by several synthetic antioxidants. In this laboratory we have demonstrated lipid peroxidation in vivo (27). High amounts of ethane (another hydroperoxide product) and malondialdehyde, respectively, were released from cercosporin-treated tobacco leaf disks and suspension cultures when they were incubated in the light. An analysis of tobacco suspension culture cells before and after cercosporin treatment showed large increases in the ratio of saturated to unsaturated fatty acids extracted from membranes of cercosporin-treated cells (29), indicating a selective destruction of the unsaturated fatty acids. Further, the addition of α-tocopherol to suspension cultures blocked the lipid peroxidation (27). Similar results were obtained by Youngman et al. (30), who demonstrated ethane release from cercosporin-treated Vicia faba protoplasts. Peroxidation of purified fatty acid methyl esters by cercosporin has also been demonstrated (27,30).

As would be expected from the above results, cercosporin-treatment of cells results in marked changes in membrane structure. Electron Spin Resonance (ESR) spectroscopy of cercosporin-treated tobacco protoplasts using two steric acid spin labels showed a marked decrease in the fluidity of the membrane as compared to untreated protoplasts (29). Along with this decrease in membrane fluidity was an apparent increase in the membrane phase transformation temperature as measured by the temperature dependence of fatty acid spin label mobility. Changes such as these have been correlated with increases in the permeability of the membrane (31), and are commonly seen in membranes damaged by peroxidation due to ozone or chemical oxidizing agents (31-34).

Although photosensitizers have many effects on cells and we cannot rule out other sites of action, the membrane-damaging activity of cercosporin appears to be a primary mechanism by which cercosporin destroys plant cells. This mode of action is also consistent with the etiology of Cercospora diseases. Since Cercospora species do not penetrate the cells of their host plants, they need a mechanism for obtaining nutrients from host cells. By breaking down the host cell membranes, cercosporin may provide the fungus with the nutrients required for growth and sporulation within the host.

Resistance Mechanisms

Mice, bacteria, and all plants which have been tested are sensitive to cercosporin (8,12-13,35), and it has not been possible to select for cercosporin-resistant cell culture mutants by mutagenesis and selection with cercosporin in tissue cultures (Daub, M.E.,

unpublished data). The fungus, however, produces high concentrations of cercosporin in the light and is apparently unaffected by it. Studies with other fungi have indicated that this resistance is not unique to Cercospora species, but is shared by a number of other fungi as well (36). Yeasts, both Saccharomyces cerevisiae and the Basidiomycete yeast Sporobolomyces, are resistant to cercosporin. Among mycelial fungi, several plant pathogens in the Ascomycete and Deuteromycete classes (for example, Alternaria, Fusarium, Colletotrichum, and Verticillium species) are also highly resistant even though there is no evidence that they produce similar toxins. By contrast, isolates of Neurospora crassa and several Aspergillus species, although taxonomically related, were sensitive to cercosporin as were fungi in the Oomycete class. The resistance of a number of different fungi to cercosporin is interesting considering the generalized toxicity of singlet oxygen and superoxide.

The current emphasis in this laboratory is to attempt to elucidate the basis of resistance of Cercospora species and other fungi to cercosporin. Our approach has been based on our knowledge of the mode of action of cercosporin and on known differences between resistant and sensitive fungi. It is important to note that Cercospora species, as well as the other fungi, are resistant to externally supplied cercosporin. Thus compartmentalization of the toxin molecule during synthesis, although possible, cannot completely account for cercosporin resistance. Detoxification of the cercosporin molecule by the fungus is also not a possibility for crystals of cercosporin are found clustered around the hyphae in culture. Finally, resistant and sensitive fungi show the same spectrum of sensitivity to hematoporhyrin, another singlet oxygen-generating photosensitizer, suggesting that the resistant fungi are able to tolerate singlet oxygen.

Several factors have been found not to be important in resistance (Daub, M. E., unpublished data). Yeasts and the higher mycelial fungi are known to have very saturated membranes as compared to those of the sensitive Oomycetes (37), but an analysis of fatty acid composition of Cercospora nicotianae demonstrated high concentrations of linoleic acid, which is sensitive to peroxidation. Also, although a superoxide dismutase—overproducing mutant of tobacco was found to have low resistance to cerosporin, oxidative enzymes do not appear to be important in fungal resistance. No significant differences were seen in superoxide dismutase activity in extracts from Cercospora species as compared to those from the sensitive fungus Phytophthora cinnamomi, and catalase activity was considerably higher in P. cinnamomi. Finally, little difference was seen in antioxidant activity of organic and aqueous extracts of sensitive and resistant fungi. By contrast, the presence of carotenoids and composition of the fungal cell wall do appear to be important in resistance.

Carotenoids. Carotenoids are highly effective quenchers of singlet oxygen and of triplet sensitizers, and appear to be the major means of defense of many organisms against photooxidations sensitized by chlorophyll and a number of photosensitizing dyes (38–39). We have found that Cercospora species produce high concentrations of

carotenoids (36). In C. nicotianae, β-carotene accounts for more than 99% of the total carotenoids present. β-carotene production peaks early in the culture cycle at approximately 12 μg/g dry weight, and then drops off, at least as a function of the dry weight increase. By contrast, two cercosporin-sensitive Phytophthora species, P. cinnamomi and P. parasitica produce about 20 ng carotenoids per gram dry weight. So there are large differences between Cercospora species and some of the cercosporin-sensitive fungi in carotenoid concentrations.

Studies with mutants of Neurospora crassa and Phycomyces blakesleeanus which are blocked in carotenoid biosynthesis support the hypothesis that carotenoids are important in fungal resistance to cercosporin. These mutants were significantly more sensitive to cercosporin than carotenoid-producing isolates (36). Since carotenoid-deficient mutants are unavailable in Cercospora, we attempted to obtain evidence for a role of carotenoids in Cercospora resistance by blocking carotenoid synthesis with several inhibitors. These included the well-characterized fungal carotenoid inhibitor diphenylamine (40), the herbicide norfluorazon which has been shown to block carotenoid synthesis in fungi as well as plants (40), and the hydroxymethyl-glutaryl-coenzyme A reductase inhibitor, mevinolin (41). In all cases, we were unsuccessful in blocking β-carotene production in C. nicotianae even though we were able to obtain 70-90% inhibition of Neurospora crassa carotenoids with the same compounds (36). At this time we cannot explain why these potent carotenoid inhibitors were effective in inhibiting carotenoid synthesis in N. crassa, but not C. nicotianae. It is not due to lack of uptake of the different inhibitors because they had comparable growth reducing effects on both fungi.

It is interesting to note that the carotenoid-producing strain of Neurospora crassa, although more resistant to cercosporin than albino isolates, is considerably more sensitive than C. nicotianae, and these two fungi produce comparable amounts of carotenoids (36). These results suggest that carotenoids are important in resistance, but that they are not the only cellular component involved. It is also possible that carotenoids play a major role in resistance of Cercospora species, but that the localization of carotenoids in the fungal cell differs between these two fungi. Localization of the carotenoids is a critical consideration. Plants, which are very sensitive to cercosporin, certainly contain carotenoids, but they are localized in chloroplasts and other plastids and thus would not be effective in protecting membranes in other parts of the cell.

Fungal Cell Wall. Another hypothesis that we have investigated is that the fungal cell wall protects the plasma membrane and other parts of the cell from cercosporin. It is possible that Cercospora species either store the toxin internally in an inactive state and activate it at the time of excretion (or compartmentalize it until excretion), and then by some means either prevent the active molecule from re-entering the cells, or quench the singlet oxygen and superoxide produced by the toxin. Since the toxin acts on membranes, the fungal cell wall would have to serve as the protective barrier.

The role of the cell wall in resistance was tested by

determining the sensitivity to cercosporin of fungal protoplasts, generated by digesting the cell walls with the enzymes cellulase, chitinase, β-glucuronidase, and amylase. Cercospora nicotianae and Neurospora crassa, although differing significantly in their resistance as mycelium, show the same highly sensitive response to cercosporin as protoplasts. For example, at 10 μM cercosporin (a concentration which is not toxic to C. nicotianae mycelium), protoplasts of both species were completely killed (42; Gwinn, K. D. and Daub, M. E., unpublished data). As the protoplasts started to regenerate cell walls, C. nicotianae protoplasts rapidly regained resistance (40% were resistant by 4 hours after isolation), whereas the N. crassa protoplasts remained very sensitive. These differences were not due to a lag in cell wall regeneration by N. crassa protoplasts. Using resistance to osmotic shock as a measure of cell wall regeneration, we found that N. crassa protoplasts actually regenerate faster; they start to become osmotically resistant at approximately 4 hours after isolation as compared to 12 hours for C. nicotianae protoplasts.

The components of the cell wall that are important in resistance have not yet been determined. In our initial studies we are looking at the regeneration of wall carbohydrates using optical brighteners and fluorescein-tagged lectins which bind to specific carbohydrate residues (Gwinn, K. D. and Daub, M. E., unpublished data). These compounds cause the protoplasts to fluoresce when they bind to them, and thus the presence of various wall components can be determined by fluoresence microscopy. Based on this technique protoplasts of the two species show the same rate of regeneration of β-glucans and of mannose, and have no detectable galactose. The binding of lectins specific for N-acetyl-glucosamine was significantly delayed in N. crassa protoplasts, suggesting that chitin deposition occurs later in this fungus than in C. nicotianae. We do not believe that the presence or absence of chitin plays any role in resistance, for both fungal species have chitin-containing walls. These data do suggest, however, that there are differences in the walls of these two fungi. Further studies are in progress.

It is important to note that even though protoplasts of C. nicotianae and N. crassa are quite sensitive to cercosporin, they are still more resistant than plant cells. Approximately 50% of freshly isolated protoplasts of both species survive treatment with 1 μM cercosporin, a concentration that is lethal to plant cells under the same light regime (Gwinn, K. D. and Daub, M. E., unpublished data). It may be that the differences in sensitivity of C. nicotianae and N. crassa to cercosporin is due to protection by the Cercospora cell wall, but the basic level of resistance found in protoplasts of both species must be due to other factors. The most likely possibility is protection by carotenoids. As stated previously, these two fungal species produce equivalent amounts of carotenoids. It is also possible that the fairly saturated membranes of these fungi are more resistant to peroxidation than the highly unsaturated membranes found in plants. Further studies are needed to elucidate all the factors involved in resistance.

Future Perspectives

Cercospora species are a rather unusual group of plant pathogens.
Whereas most plant pathogenic fungi have fairly restricted host
ranges, Cercospora species attack a vast number and diversity of
hosts. Further, it has been hard to control these pathogens, due to
the difficulty in identifying adequate levels of resistance in
natural populations of host plants. Much of the success of this
group of pathogens may be due to their production of the phytotoxin,
cercosporin. No plants which show resistance to cercosporin have
been identified, and it has not been possible to induce even
moderate levels of cercosporin resistance by mutagenesis and
selection of plant cells with cercosporin in culture. Cercospora
species may in fact be successful because they have the ability to
produce a molecule against which plants cannot defend themselves.
Although photoactivated toxins have not been known to play a role in
other plant diseases, compounds of similar structure have been
isolated from species of Alternaria and Cladosporium (43-45),
suggesting that photosensitization may be an important mechanism of
pathogenesis for some fungi.

It is hoped that studies on resistance of fungi to cercosporin
will be useful in developing new and better ways of controlling
diseases caused by Cercospora species and perhaps other plant
pathogenic fungi as well. If in fact it is not possible to induce
resistance to the toxin in host plants, perhaps the problem can be
approached from an opposite, but equally effective angle, that is,
disruption of the resistance mechanisms of the fungus itself. This
could be accomplished by the use of new non-toxic chemical control
targeted at disrupting a resistance mechanism rather than killing
the fungus. Alternatively, it may be possible to selectively breed
or genetically engineer plants for the production of compounds that
disrupt the fungal defense mechanisms. The possibility of
genetically modifying plants not to resist pathogens, but to disrupt
their virulence mechanisms, is an intriguing one that holds promise
for future disease control strategies.

Literature Cited

1.   Main, C. E.; Byrne, S. V. (eds.). 1985 Estimates of Crop
      Losses in North Carolina Due to Plant Diseases and Nematodes;
      Dept. Plant Pathology Special Publication No. 5, North
      Carolina State University, Raleigh, N.C., 1986; p 34.
2.   Calpouzos, L. Ann. Rev. Phytopathol. 1966, 4, 369-90.
3.   Calpouzos, L.; Stallknecht, G. F. Phytopathology 1967,
      57, 799-800.
4.   Meredith, D. S. Banana Leaf Spot Disease (Sigatoka) caused
      by Mycosphaerella musicola Leach; Commonwealth Mycological
      Institute:Kew, Surrey, England, 1970.
5.   Thorold, C. A. Trop. Agric. Trin. 1940, 17, 213-14.
6.   Lousberg, R.J.J.Ch.; Weiss, U.; Salemink, C. A.; Arnone, A.;
      Merlini, L.; Nasini, G. Chem. Commun. 1971, 1971, 1463-64.
7.   Yamazaki, S.; Ogawa, T. Agric. Biol. Chem. 1972, 36, 1707-
      18.

8. Yamazaki, S.; Okubo, A.; Akiyama, Y.; Fuwa, K. Agric. Biol. Chem. 1975, 39, 287-88.
9. Deutschmann, F. Phytopathol. Z. 1953, 20, 297-310.
10. Kuyama, S.; Tamura, T. J. Amer. Chem. Soc. 1957, 59, 5725-26.
11. Assante, G.; Locci, R.; Camarda, L.; Merlini, L.; Nasini, G. Phytochemistry 1977, 16, 243-47.
12. Balis, C.; Payne, M. G. Phytopathology 1971, 61, 1477-84.
13. Fajola, A. O. Physiol. Plant Pathol. 1978, 13, 157-64.
14. Lynch, F. J.; Geoghegan, M. J. Trans. Brit. Mycol. Soc. 1977, 69, 496-98.
15. Mumma, R. O.; Lukezic, F. L.; Kelly, M. G. Phytochemistry 1973, 12, 917-22.
16. Venkataramani, K. Phytopathol. Z. 1976, 58, 379-82.
17. Nasini, G.; Merlini, L.; Andreettii, G. D.; Bocelli, G.; Sgarabotto, P. Tetrahedron 1982, 38, 2787-2796.
18. Okubo, A.; Yamazaki, S.; Fuwa, K. Agr. Biol. Chem. 1975, 39, 1173-75.
19. Lynch, F. J.; Geoghegan, M. J. Trans. Brit. Mycol. Soc. 1979, 73, 311-327.
20. Macri, F.; Vianello, A. Plant Cell Environ. 1979, 2, 267-71.
21. Daub, M. E. Phytopathology 1982, 72, 370-74.
22. Daub, M.E.; Hangarter, R. P. Plant Physiol. 1983, 73, 855-57.
23. Dobrowolski, D. C; Foote, C. S. Angewante Chemie 1983, 95, 729-30.
24. Furusawa, I.; Tanaka, K.; Thanutong, P.; Mizuguchi, A.; Yazaki, M.; Asada, K. Plant Cell Physiol. 1984, 25, 1247-54.
25. Steinkamp, M. P.; Martin, S. S.; Hoefert, L. L.; Ruppel, E. G. Physiol. Plant Pathol. 1979, 15, 13-26.
26. Steinkamp, M. P.; Martin, S. S.; Hoefert, L. L.; Ruppel, E. G. Phytopathology 1981, 71, 1272-81.
27. Daub, M. E. Plant Physiol. 1982, 69, 1361-64.
28. Cavallini, L.; Bindoli, A.; Macri, F.; Vianello, A. Chem. Biol. Interactions 1979, 28, 139-46.
29. Daub, M. E.; Briggs, S. P. Plant Physiol. 1983, 71, 763-66.
30. Youngman, R. J.; Schieberle, P.; Schnabl, H.; Grosch, W.; Elstner, E. F. Photobiochem. Photobiophys. 1983, 6, 109-19.
31. Kunimoto, M.; Inoue, K.; Nojima, S. Biochim. Biophys. Acta 1981, 646, 169-78.
32. Chia, L. S.; Thompson, J. E.; Dumbroff, E. B. Plant Physiol. 1981, 67, 415-20.
33. Dobretsov, G. E.; Borschevskaya, T. A.; Petrov, V. A.; Vladimirov, Y. A. FEBS Lett. 1977, 84, 125-28.
34. Pauls, K. P.; Thompson, J. E. Physiol. Plant. 1981, 53, 255-62.
35. Lynch, F. J.; Geoghegan, M. J. Trans. Brit. Mycol. Soc. 1979, 72, 31-37.
36. Daub, M. E.; Payne, G. A. Phytopathology 1985, 75, 1298 (Abstr.)
37. Wassef, M. K. Adv. Lipid Res. 1977, 15, 159-232.
38. Krinsky, N. I. Pure Appl. Chem. 1979, 51, 649-660.
39. Foote, C. S.; Denny, R. W.; Weaver, L.; Chang, Y.; Peters, J. Ann. N. Y. Acad. Sci. 1970, 171, 139-145.

40. Ruddat, M.; Garber, E. D. In Secondary Metabolism and
     Differentiation in Fungi; Bennett, J. W.; Ciegler, A., Eds.;
     Marcel Dekkar Inc.: New York, 1983; Chapter 5.
41. Endo, A.; Kuroda, M.; Tanzawa, K. FEBS Lett. 1976, 72, 323-
     326.
42. Gwinn, K. D.; Daub, M. E. Phytopathology 1985, 75, 1298
     (Abstr.).
43. Overeem, J. C.; Sijpesteijn, A. K. Phytochemistry 1967, 6, 99-
     105.
44. Yoshihara, T.; Shimanuki, T.; Araki, T.; Sakamura, S.
     Agric. Biol. Chem. 1975, 39, 1683-84.
45. Robeson, D.; Strobel, G.; Matsumoto, G. K.; Fisher, E. L.;
     Chen, M. H.; Clardy, J. Experientia 1984, 40, 1248-50.

Chapter 21

Light-Activated Antimicrobial Chemicals from Plants: Their Potential Role in Resistance to Disease-Causing Organisms

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A variety of plants produce chemical constituents
which are effective, light-activated antimicrobials in
vitro. The role of such natural products in situ
remains unclear, but their potent biocidal activity
suggests that they may represent an important
biochemical defense against pathogenic organisms. The
first part of this chapter is used to examine what is
known concerning the toxicity of furanocoumarin,
polyacetylene and pterocarpan phototoxins toward
various plant pathogens. Results of recent studies in
which the susceptibility of nine fungal pathogens of
the genus Citrus (Family Rutaceae) to leaf extracts
and to various coumarins isolated from three Citrus
species (C. limettoides, C. macrophylla and C. medica)
are discussed in the latter section.

In a recent volume on plant disease, Cowling and Horsfall ([1])
compared plant defense with the defense of a medieval castle. They
pointed out that higher plants, like castles, are immobile and must
be prepared to protect themselves from would-be attackers (i.e.,
herbivores and potential pathogens) whenever challanged. The first
line of defense against invading armies for castle dwellers was an
outer castle wall. External plant surfaces (e.g., the cuticle,
epidermal cells, bark, etc.) serve a similar function and generaly
preclude ·pathogenic organisms from gaining entrance to internal
tissues. A variety of measures within castles were also in place to
defend castle inhabitants should the outer walls be breached. Mazes
of rooms and corridors, trap doors and doors with sturdy locks, as
well as the occasional secret passage were intended to impede the
progress of invading armies and allow the castle defenders time to
regroup or escape. Plants also have elaborate internal defenses
which effectively prevent penetration by potential pathogens. Such
defenses include a variety of physical barriers, e.g., cellulosic
walls, lignified and suberized tissues, as well as a diverse array
of toxic biochemicals.

Endogenous antimicrobials are among the most actively studied
of the various biochemical defenses evolved by plants (2-7). In
this chapter, we will review what is currently known about the
involvement of several classes of light-activated or "solar-powered"
antimicrobials in plant resistance to disease-causing organisms.
The results of recent work with Citrus "phototoxins" or
"photosensitizers" and their effects on fungal pathogens of that
genus also will be described. Finally, phytochemical classes which
may mediate phototoxic antimicrobial activity, but have yet to be
examined for such biological action, will be pointed out.

Photosensitizers As Plant Defensive Agents

Plant metabolites from at least ten different phytochemical classes
are capable of light-enhanced toxicity and may be synthesized by
species in as many as ten percent of all living plant families (8).
The chemistry, distribution, toxicology and ecological importance of
many of these biologically active metabolites are discussed in
several recent reviews (8-11); however, these reports pay little
attention to the potential importance of endogenous phototoxins in
plant defense against disease-causing organisms. The involvement of
various phototoxic antimicrobial furanocoumarins, polyacetylenes and
pterocarpans in plant resistance are considered separately below.

Furanocoumarins. Psoralens or furanocoumarins occur widely in the
plant kingdom and are characteristic constituents in the Apiaceae
(Umbellifereae), Fabaceae (Leguminosae), Moraceae and Rutaceae among
other families (12,13). Approximately 120 different derivatives
have been isolated and identified (13); many are potent phototoxins
capable of killing or inhibiting the growth of viruses, bacteria and
fungi as well as affecting a broad-spectrum of higher organisms (see
ref. 9 for review). Photoactive furanoucoumarins require excitation
by near ultraviolet or UVA wavelengths (320-400 nm) for full
expression of their toxic action, although light-independent affects
are also known (see discussion by Ivie, this volume). Covalent
bonding to DNA presumably accounts for most of their phototoxic
consequences (14); however, photooxidative cellular damage
(resulting from excited oxygen species) and photobinding to cellular
proteins have also been demonstrated (15-17).
Furanocoumarins are generally present in healthy plant tissues
(13,18) where they may function as preformed or preinfectional
antimicrobials which inhibit the establishment of disease-causing
organisms. Suprisingly little work has been conducted on the
toxicity of psoralens toward plant viruses, phytopathogenic bacteria
or fungi which might encounter these metabolites in situ despite our
understanding of their phototoxicity toward other organisms. The
bacterium Agrobacterium tumefacians and members of several fungal
genera including Botrytis, Ceratocystis, Sclerotinia, Stereum and
Gleosporium are among the phytopathogenic organisms known to be
affected by various furanocoumarins (19-23).
The accumulation or enhanced biosynthesis of furanocoumarins in
plant tissues and cell suspension cultures exposed to biotic and
abiotic stresses suggests their potential for involvement in
postinfectional plant responses. The concentration of xanthotoxin

or 8-methoxypsoralen (8-MOP; II), one of the most frequently
encountered furanocoumarin photosensitizers, dramatically increases
in diseased carrot tissue (Daucus carota) (24), in parsnip
(Pastinaca sativa) root discs inoculated with Ceratocystis fimbriata
as well as other nonpathogens of parsnip (21) and in celery (Apium
graveolans) following infection with the "pink-rot" fungus
Sclerotinia sclerotiorum (19). The biosynthesis and accumulation of
other phototoxic furanocoumarins including bergapten or
5-methoxypsoralen (5-MOP; I) (in carrot and celery) and
4,5',8-trimethylpsoralen (in celery) are also induced following
infection by disease-causing organisms (24,25). Cell suspension
cultures of parsely (Petroselinum hortense) exposed to fungal
elicitors accumulate 8-MOP in a simiar response (26,27).

Polyacetylenes and Their Derivatives. Polyacetylenes and their
derivatives represent one of the largest and most exhaustively
studied classes of plant metabolites. More than 700 different
structures ranging from straight-chain polyacetylenic molecules to
various ring stabilized structures are included in this class (28).
These phytochemicals occur primarily among members of the Apiaceae
(Umbelliferae), Araliaceae, Asteraceae (Compositae), Campanulaceae,
Pittosporaceae, Oleaceae and Santalaceae (28). At least two other
families, the Fabaceae (Leguminosae) and the Solanaceae, have
members that produce acetylenes; however these metabolites are
synthesized only in response to infection by pathogenic organisms
(29,30).
    Many polyacetylenes are potent photosensitizers which can enter
an excited state following absorption of light energy. In this
excited state, such molecules mediate a variety of broad-spectrum
phototoxic responses (see 9-11 for reviews). Two modes of action
have been suggested. Straight-chain acetylenic molecules tend to
interact directly with target biomolecules in cells through radical
mechanisms (31,32) while thiophenes, sulfur-derivatives of various
acetylenic precursors, mediate the oxidation of a variety of
biomolecules (membrane lipids and proteins in particular)
presumably via singlet oxygen generation (32-34). These
contrasting mechanisms apparently compete in other ring stabilized
acetylenes (32). Light-independent toxicity has been noted for a
variety of acetylenic metabolites (31,35); however the actual
mechanisms involved in such interactions have not been adaquately
studied.
    The involvement of polyacetylenic molecules in plant disease
resistance has received considerable attention. At least 15
phytochemicals from this class have presumed roles as pre- and/or
postinfectional antimicrobial agents (Table I). Several
polyacetylenes and thiophenes isolated from members of the
Asteraceae may function as preformed antibiotics. Such a role is
suggested by their potent phototoxicity toward phytopathogens
(8,41,43) and because they can be isolated from healthy plant
tissues (28,53,54). Other studies report that the biosynthesis of
acetylenes in whole plants and in tissue cultures can be stimulated
by various factors (42,43,55) which may indicate their potential
involvement in postinfectional defensive responses. Kourany (43)
investigated the fate of various acetylenic thiophenes in Tagetes
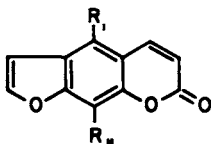
I. **5-Methoxypsoralen**; $R_I = OMe$, $R_{II} = H$

II. **8-Methoxypsoralen**; $R_I = H$, $R_{II} = OMe$

III. **5,8-Dimethoxypsoralen**; $R_I = OMe$, $R_{II} = OMe$

Table 1. Plants which contain antifungal polyacetylenes indicated
in disease resistance

Plant Source	Polyacetylene	References
Aegopodium podagraria L. (Apiaceae)	Heptadeca-1,9-diene-4,6-diyne-3-ol (falcarinol)	29,36-38
Daucus carota (Apiaceae) Lycopersicon esculentum (Solanaceae)	Heptadeca-1,9-diene-4,6-diyne-3,8-diol (falcarindiol)	
Dendropanax trifidus Makino (Araliaceae)	16-Hydroxyoctadeca-9,17-diene-12,14-diynoic acid Octadeca-9,17-diene-12,14-diyn-1,16-diol	39
Bidens pilosa L. (Asteraceae)	1-Phenylhepta-1,3,5-triyne	40,41
Tagetes erecta L. Tagetes patula L. (Asteraceae)	2,2':5',2"-Terthienyl 5-(4-Hydroxy-1-butenyl)-2, 2'-bithienyl 5-(4-Acetoxy-1-butenyl)-2, 2'-bithienyl 5-(Buten-3-ynyl)-2, 2'-bithienyl	8,42,43
Lycopersicon esculentum .	Tetradeca-6-ene-1,3-diyne-5,8-diol	29,44
Carthamus tinctoris (Asteraceae)	Trideca-3,11-diene-5,7,9-triyne-1,2-diol (safynol) Trideca-11-ene-3,5,7,9-tetrayne-1,2-diol (dehydrosafynol)	45-48
Lens culinaris (Fabaceae) Lens nigrricans Vicia faba (Fabaceae) + 31 other Vicia species	Wyerone Wyerone Acid Wyerone Epoxide	30,49-52

erecta L. (the african marigold) in response to exposure to several
pathogens. Inoculation of seedlings with highly pathogenic strains
of Alternaria tagetica and Fusarium oxysporum f.sp. radicis
lycoperisici led to a general decrease in thiophene levels compared
to control plants. Infection of plants with Fusarium oxysporum var.
callistephi race 2, a moderately virulent pathogen, resulted in
accumulation of alpha-terthienyl (IV) and two bithiophene
derivatives above levels encountered in non-infected plants.   In a
related species, dwarf marigold (Tagetes patula L.) plants and
tissue cultures infected or transformed with Agrobacterium
tumefaciens also accumulated thiophenes (42).   These studies suggest
that thiophene biosynthesis can be stimulated by moderately virulent
pathogens (which may lead to increased plant resistance to
infection), but that highly pathogenic species may avoid this plant
response by suppressing the production of these toxic biochemicals.
Phenylheptatriyne (PHT, V), a phototoxic polyacetylene which occurs
in healthy tissues of Bidens pilosa L. (Asteraceae), may function as
a preinfectional inhibitor similar to the thiophenes of T. erecta
discussed above (41). Recent work with tissue cultures of B. pilosa
indicates that synthesis also may be stimulated by a fungal
culture-filtrate (55).

   Three diacetylene alcohols [falcarinol (VI), falcarindiol and
tetradeca-6-ene-1,3-diyne-5,8-diol], two triacetylene alcohols
[dehydrosafynol (VII) and safynol] and three furanoacetylenes
[wyerone (VIII), wyerone acid and wyerone epoxide] are also
important antifungal metabolites implicated in induced resistance
responses in plants (30,46-48,50,56). Falcarinol and falcarindiol
occur in healthy tissue of Falcaria vulgaris (28), Daucus carota
(37) and Aegopodium podagraria (57). These molecules also
accumulate rapidly in carrot root tissue following wound-inoculation
with Botrytis cinerea (38) and in tomato infected with Cladosporium
fulvum (29). A third linear acetylene, cis-tetradeca-6-ene-1,
3-diyne-5,8-diol, co-ocurrs with falcarinol and falcarindol in
diseased tomato (44). Safynol and dehydrosafynol, two triacetylene
alcohols with pronounced antifungal activity, occur in safflower
(Carthamus tinctoris) (45-47). These particular metabolites are
rapidly biosynthesized by this plant in reponse to infections with a
virulent strain of Phytophthora drechsleri and an avirulent strain
of P. megasperma var. sojae. Within 48 h of inoculation, the levels
of safynol and dehydrosafynol may increase by as much as 40 and
1,500 times, respectively (48). The rate of dehydrosafynol
accumulation is statistically correlated with high disease
resistance in one particular breeding line (Biggs) of safflower
(48). Wyerone occurs in healthy tissues of the broad bean Vicia
faba L. (49) and accumulates, usually in conjunction with wyerone
epoxide, in at least 28 other species of Vicia and two species of
Lens when challenged by Helminthosporium carbonum or Botrytis
cinerea (30). Wyerone acid co-occurs with wyerone and wyerone
epoxide in broad bean plants infected with species of Botrytis (52).

   Despite their structural similarity with other phototoxic
acetylenes, falcarinol and falcarindiol (and closely related
metabolites like falcarinone and falcarindione), apparently are not
phototoxic (9). Whether the antimicrobial activity of
tetradeca-6-ene-1,3-diyne-5,8-diol, safynol, dehydrosafynol or the

wyerone derivatives may result from light-activated or
light-independent processes remains a critical point that needs to
be established.

Pterocarpans. Several pterocarpan derivatives, most often referred
to in the literature as isoflavonoid phytoalexins, predominantly
occur in members of the Fabaceae (Leguminosae) (30). Like
furanocoumarins and polyacetylenes, these phytochemicals are toxic
toward a wide range of biological organisms and accumulate in plant
tissues in response to a variety of stresses (particularly infection
by pathgens) (30,58). For these reasons, certain pterocarpans are
believed to play an important role in the defense of producing
plants against potential disease-causing organisms. The
biosynthesis, elicitation and biological activity of isoflavonoid
phytoalexins has been reviewed quite recently (58) and will be
discussed only briefly here.
        Considerable interest has been focused on an array of
antimicrobial pterocarpan derivatives because of their putative
involvement in plant defense. A large amount of information is
available concerning the cellular targets and modes of action of
these plant metabolites (58). Bacterial and fungal membranes are
particularly susceptible to the effects of isoflavonoid
phytoalexins; however, other cellular sites have not been ruled out
as targets of action (58). One investigation has examined the
involvement of light as an activating factor in pterocarpan
toxicity. Bakker et al. (59) found that several pterocarpan
derivatives including glyceollin I (IX), phaseollin (X) and pisatin
(XI) as well as 3,6a,9-trihydroxypterocarpan and tuberosin can form
free radicals in the presence of UV irradiation (with maximum
intensity around 305 nm) and that these free radicals are most
likely involved in the inactivation of glucose-6-phosphate
dehydrogenase activity in vitro. The extent to which free radical
formation may contribute to the pterocarpan toxicity (and
presumably plant defense) in other studies where the effect of light
was not considered is not clear, but certainly warrants further
attention.

Recent Investigations

Despite demonstrations that various phytochemicals are potent
light-activated antimicrobials in vitro and that many also
accumulate in response to infection by disease-causing organisms or
other stressful situations, there is little direct evidence linking
such molecules to plant defense in situ. We have been studying the
role of endogenous photosensitizers to determine their involvement
in the resistance of Citrus species to disease-causing organisms
since finding that the leaves of many species contain
photosensitizers (8). Our efforts thus far have concentrated on: 1)
establishing the susceptibility of various Citrus pathogens to leaf
extracts; 2) identifying the phototoxic phytochemicals in these leaf
extracts; and 3) determining pathogen susceptibility to the
phytochemicals responsible for this biocidal action.
        Initially, we were interested in determining the susceptibility
of fungal pathogens isolated from Citrus to leaf extracts previously

shown to elicit phototoxic activity (8). Nine disease-causing fungi
were obtained for these studies (Table II) including various root,
fruit and/or leaf pathogens. Susceptibility was determined by
screening the pathogenic organisms against extracts of Citrus
limettoides Tan. (sweet lime), C. macrophylla Wester (alemow) and C.
medica L. (citron) using a disc bioassay. Plant extracts were
prepared by homogenizing freshly collected leaf material (100 g) in
methanol (300 ml) followed by filtration and concentration of the
extract to a final volume of 10 ml. Sterile filter paper discs were
loaded with the different extracts (20 ul) and allowed to dry. The
discs were placed onto duplicate potato dextrose agar (PDA) plates
containing either spores or mycelial fragments of the nine
phytopathogenic organisms and then incubated in the dark for 60 min.
One of the duplicate plates was irradiated for 2 h with UVA (2 W
m^{-2}) while the other plate was kept in the dark to monitor
light-independent antimicrobial action. All plates were
subsequently incubated in the dark (at 25°C for 24-48 h) and
then scored for zones of inhibition surrounding the filter paper
discs.

Table II.   Fungal pathogens of Citrus

Leaf Pathogens
    Alternaria citri - leafspot
    Colletotrichum gleosporides - anthracnose
Fruit Pathogens
    Alternaria citri - black rot
    Colletotrichum gleosporides - anthracnose
    Diploidia natalensis - stem-end rot
    Geotrichum candidum - sour rot
    Penicillium digitatum - green mold
    Penicillium italicum - blue mold
Root Pathogens
    Fusarium oxysporum - root rot
    Fusarium solani - root rot
    Phytophthora parasitica - foot rot

Four of the fungi tested were quite sensitive to the Citrus
extracts in the presence of UVA, but were unaffected in its absence
(Table III). Three of the susceptible pathogens primarily infect
roots (F. oxysporum, F. solani and Phytophthora parasitica) while
the fourth, Colletotrichum gleosporidies, infects mainly leaves and
fruit. Other pathogens of above-ground plant parts, namely A.
citri, D. natalensis, G. candidum and P. digitatum, successfully
resisted the light-activated antimicrobial action of all three
extracts. P. italicum, however, was slightly affected by the Citrus
macrophylla extract.
    Five coumarin derivatives were identified in leaf extracts of
C. limettoides, C. macrophylla and C. medica including
5-hydroxypsoralen, 5-methoxypsoralen (I), 5,8-dimethoxypsoralen
(III), 4-hydroxycoumarin and 7-hydroxycoumarin [8-MOP although
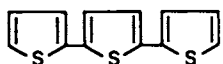
IV. Alpha-Terthienyl

V. Phenylheptatriyne

$$H_2C = CH - CH - C \equiv C - C \equiv C - CH_2 CH = CH - (CH_2)_6 - CH_3$$
$$\quad\quad\quad |$$
$$\quad\quad\quad OH$$

VI. Falcarinol

$$HOH_2C - CH - C \equiv C - C \equiv C - C \equiv C - C \equiv C - CH = CH - CH_3$$
$$\quad\quad\quad |$$
$$\quad\quad\quad OH$$
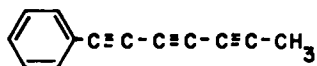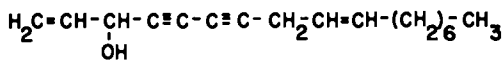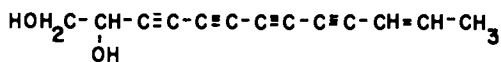
VII. Dehydrosafynol

$$CH_3 - CH_2 - CH = CH - C \equiv C - C - \overset{O}{\underset{\|}{C}} \quad CH = CH - CO_2 Me$$
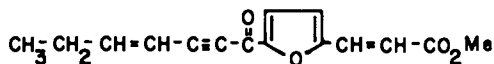
VIII. Wyerone

IX. Glyceollin I
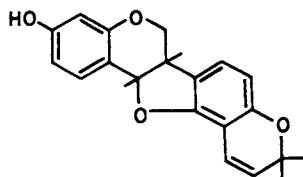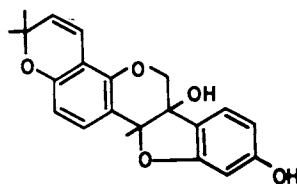
X. Phaseollin

XI. Pisatin
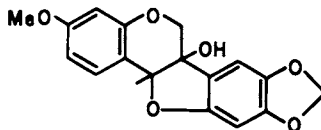
Table III. UVA-Induced inhibitory effect of crude leaf extracts on the growth of <u>Citrus</u> pathogens

	Inhibition Zones					
	<u>C. limettoides</u>		<u>C. macrophylla</u>		<u>C. medica</u>	
Pathogens	Dark	UVA	Dark	UVA	Dark	UVA
Alternaria citri	-	-	-	-	-	-
Colletotrichum gleosporides	-	++	-	++	-	++
Diploidia natalensis	-	-	-	-	-	-
Fusarium oxysporum	-	++	-	++	-	++
Fusarium solani	-	++	-	++	-	++
Geotrichum candidum	-	-	-	-	-	-
Penicillium digitatum	-	-	-	+	-	-
Penicillium italicum	-	-	-	-	-	-
Phytophthora parasitica	-	++	-	++	-	++

- No inhibition.
+ Inhibition zones below 10 mm.
++ Inhibition zones between 11 - 15 mm.
+++ Inhibition zones betweem 16 - 20 mm.

common in closely related genera has not been reported in Citrus
(13)]. The phototoxicities of these derivatives were tested against
the nine pathogens listed in Table II. The same bioassay procedures
as above were used except that the individual chemicals (dissolved
in methanol) were applied to the filter paper discs instead of leaf
extracts. Only 5-methoxypsoralen (5-MOP) elicited phototoxic
responses (Table IV). D. natalensis and the two Penicillium species
were resistant in these in vitro bioassays.

     In general, these studies suggest that fungi which infect
above-ground plant tissues are more resistant to phototoxic action
than root pathogens. Such contrasting responses probably reflect an
evolved ability by certain leaf and fruit pathogens to circumvent
the toxic action of these chemicals via detoxification or other
processes. Since UVA is rarely experienced in the rhizosphere,
evolution of such processes by root pathogens would seem to be
unnecessary. Colletotrichum gleosporides appears to be an
exception. Unlike the other pathogens of above-ground tissues, this
fungus was susceptibile to 5-MOP and all three of the Citrus
extracts which suggests that establishment of this fungus on plant
tissues may be influenced by the presence of endogenous
photosensitizers.

     We have isolated several other phototoxic coumarins from
various Citrus species using standard bioassay organisms (i.e., E.
coli and Saccharomyces cerevisiae). In addition to 5-MOP, the
chemicals 7-methoxycoumarin and 5-geranoxypsoralen (bergamottin)
have also been identified. The toxicity of these metabolites have
not yet been established using the Citrus pathogens. Once this has
been accomplished, we intend to quantitate the levels of endogenous
photosensitizers in field and in greenhouse-grown Citrus plants and
establish whether plant resistance to pathogen infection can be
correlated with in situ levels of particular photosensitizers.

Conclusion

We have discussed the antimicrobial activity of more than 20
phytochemicals; most are potent phototoxins. Others are included,
not because they are demonstrated photosensitizers, but because they
share common chemical characteristics with these biologically active
plant metabolites, i.e., extensive aromatic or conjugated double
and/or triple bond systems, and may function similarly in vivo. In
addition to the molecules already discussed, numerous other
plant-derived photosensitizers are known, but their role in
plant-pathogen interactions have yet to be established. Included
are various acetophenone, extended quinone, furanochromone and
lignan derivatives as well as several beta-carboline,
furanoquinoline and isoquinoline alkaloids (8,9). Other metabolites
which are involved with plant responses to pathogenic invasion and
have similar structural features have recently been isolated, e.g.,
naphthofuranones (60) and dibenzofurans (61-63), and warrant further
investigation with regard to their potential phototoxic action.

     Important areas for future research that might aid in further
elucidating the significance of photosensitizers in plant defense
against disease-causing organisms include among others: 1)
toxicological studies to determine cellular mechanisms of pathogen

Table IV. Inhibitory effect of various coumarins and furanocoumarins on Citrus pathogens

Pathogens	4-HC		7-HC		5-HP		5-MOP		5,8-diMOP	
	Dark	UVA	Dark	UVA	Dark	UVA	Dark	UVA	Dark	UVA
Alternaria citri	–	–	–	–	–	–	–	++	–	–
Colletotrichum gleosporides	–	–	–	–	–	–	–	++	–	–
Diploidia natalensis	–	–	–	–	–	–	–	–	–	–
Fusarium oxysporum	–	–	–	–	–	–	–	++++	–	–
Fusarium solani	–	–	–	–	–	–	–	++	–	–
Penicillium digitatum	–	–	–	–	–	–	–	–	–	–
Penicillium italicum	–	–	–	–	–	–	–	–	–	–
Phytophthora parasitica	–	–	–	–	–	–	–	++	–	–

– No inhibition.
+ Inhibition zones below 10 mm.
++ Inhibition zones between 11 - 15 mm.
+++ Inhibition zones between 16 - 20 mm.
++++ Inhibition zones above 21 mm.

4-HC – 4-hydroxycoumarin
7-HC – 7-hydroxycoumarin
5-HP – 5-hydroxypsoralen
5-MOP – 5-methoxypsoralen
5,8-MOP – 5,8-dimethoxypsoralen

susceptibility (or resistance) to phototoxic metabolites; 2)
phytochemical studies to evaluate the quantitative variation of
specific phototoxins in plant populations coupled with in situ
studies that attempt to correlate those endogenous levels with plant
resistance (or susceptibility) to specific pathogens; and 3)
breeding studies to select for plant lines that synthesize a
gradient of endogenous photosensitizer concentrations which can be
evaluated for resistance to a broad range of virulent organisms. In
addition, past studies that involved plant photosensitizers, but did
not consider light as an activating element in their toxicity, need
to be re-evaluated.

Literature Cited

1. Cowling, E.B.; Horsfall, J.G. In Plant Disease An Advanced
    Treatise. How Plants Defend Themselves; Horsfall, J.C.;
    Cowling, E.B., Eds.; Academic: New York, 1980, Vol. V,
    pp. 1-16.
2. Overeem, J.C. In Biochemical Aspects of Plant-Parasite
    Relationships; Friend, J.; Threlfall, D.R., Eds.; Academic: New
    York, 1976, pp. 195-206.
3. Kuc, J.; Shain, L. In Antifungal Compounds. Interactions in
    Biological and Ecological Systems; Siegel, M.R.; Sisler, H.D.,
    Eds.; Marcel Dekker: New York, 1977, Vol 2, pp. 497-535.
4. Swain, T. Ann. Rev. Plant Physiol. 1977, 28, 479-501.
5. Bell, A.A. Ann. Rev. Plant Physiol. 1981, 32, 21-81.
6. Smith, D.A. In Phytoalexins; Bailey, J.A.; Mansfield, J.W.,
    Eds.; Wiley: New York, 1982, pp. 218-52.
7. Darvill, A.G.; Albersheim, P. Ann. Rev. Plant Physiol. 1984,
    35, 243-75.
8. Downum, K.R. In Natural Resistance of Plants to Pests: Roles of
    Allelochemicals; Green, M.B.; Hedin, P.A., Eds.; ACS SYMPOSIUM
    SERIES No. 296, American Chemical Society: Washington, D.C.,
    1986, pp. 197-205.
9. Towers, G.H.N. Can. J. Bot. 1984, 62, 2900-11.
10. Knox, J.P.; Dodge, A.D. Phytochem. 1985, 24, 889-96.
11. Downum, K.R.; Rodriguez, E. J. Chem. Ecol. 1986, 12, 823-34.
12. Gray, A.I.; Waterman, P.G. Phytochem. 1978, 17, 845-64.
13. Murray, R.D.H.; Mendez, J.; Brown, S.A. The Natural Coumarins:
    Occurrence, Chemistry and Biochemistry; Wiley: New York, 1982.
14. Song, P.-S.; Tapley, K.J., Jr. Photochem. Photobiol. 1979, 29,
    1177-97.
15. Veronese, F.M.; Schiavon, O.; Bevilacqua, R.; Bordin, F;
    Rodighiero, G. Photochem. Photobiol. 1982, 36, 25-30.

16. Granger, M.; Helene, C. Photochem. Photobiol. 1983, 38, 563–68.
17. Tuveson, R.W.; Berenbaum, M.R.; Heininger, E.E. J. Chem. Ecol. 1986, 12, 933–48.
18. Beier, R.C.; Ivie, G.W.; Oertli, E.H.; Holt, D.L. Fd. Chem. Toxic. 1983, 21, 163–65.
19. Scheel, L.D.; Perone, V.B.; Larkin, R.L.; Kupel, R.E. Biochem. 1963, 2, 1127–31.
20. Martin, J.T.; Baker, E.A.; Byrde, R.J.W. Ann. Appl. Biol. 1966, 57, 491–500.
21. Johnson, C.; Brannon, D.R.; Kuc, J. Phytochem. 1973, 12, 2961–62.
22. Martin, J.T. In Fungal Pathogenicity and the Plant's Response; Byrde, R.J.W.; Cutting, C.V., Eds.; Academic: New York, 1973, pp. 333–5.
23. Afek, U.; Sztejnberg, A. Phytochem. 1986, 25, 1855–56.
24. Ceska, O.; Chaudhary, S.K.; Warrington, P.J.; Ashwood-Smith, M.J. Phytochem. 1986, 25, 81–83.
25. Wu, C.M.; Koehler, P.E.; Ayres, J.C. Appl. Microbiol. 1972, 23, 852–56.
26. Chappel, J; Hahlbrock, K. Nature 1984, 311, 76–78.
27. Hauffe, K.D.; Hahlbrock, K.; Scheel, D. Z. Naturforsch. 1986, 41c, 228–39.
28. Bohlmann, F; Burkhardt, T; Zdero, C. Naturally Occurring Acetylenes; Academic: London, 1973.
29. de Wit, P.J.G.M.; Kodde, E. Physiol. Plant Path. 1981, 18, 143–48.
30. Robeson, D.J.; Harborne, J.B. Phytochem. 1980, 19, 2359–65.
31. McLachlan, D.; Arnason, J.T.; Lam, J. Photochem. Photobiol. 1984, 39, 177–82.
32. McLachlan, D.; Arnason, T.; Lam, J. Biochem. System. Ecol. 1986, 14, 17–23.
33. Arnason, T.; Chan, G.F.Q.; Wat, C.-K.; Downum, K.R.; Towers, G.H.N. Photochem. Photobiol. 1981, 33, 821–24.
34. Downum, K.R.; Hancock, R.E.W.; Towers, G.H.N. Photochem. Photobiol. 1982, 36, 517–23.
35. Champagne, D.E.; Arnason, J.T.; Philogene, B.J.R.; Morand, P.; Lam, J. J. Chem. Ecol. 1986, 12, 835–58.
36. Kemp, M.S. Phytochem. 1978, 17, 1002.
37. Garrod, B.; Lewis, B.G.; Coxon, D.T. Physiol. Plant Path. 1978, 13, 241–46.
38. Harding, V.K.; Heale, J.B. Physiol. Plant Path. 1980, 17, 277–89.
39. Hansen, L.; Boll, P.M. Phytochem. 1986, 25, 285–93.
40. DiCosmo, F.; Towers, G.H.N.; Lam, J. Pestic. Sci. 1982, 13, 589–94.
41. Bourque, G.; Arnason, J.T.; Madhosingh, C.; Orr, W. Can. J. Bot. 1985, 63, 899–902.
42. Norton, R.A.; Finlayson, A.J.; Towers, G.H.N. Phytochem. 1985, 24, 719–22.
43. Kourany, E. M.S. Thesis, University of Ottawa, 1986, 170 pp.
44. Elgersma, D.M.; Overeem, J.C. Neth. J. Plant Path. 1981, 87, 69–70.
45. Thomas, C.A.; Allen, E.H. Phytopath. 1970, 60, 261–63.
46. Allen, E.H.; Thomas, C.A. Phytochem. 1971, 10, 1579–82.

47. Allen, E.H.; Thomas, C.A. Phytopath. 1971, 61, 1107-09.
48. Allen, E.H.; Thomas, C.A. Phytopath. 1972, 62, 471-74.
49. Fawcett, C.H.; Spencer, D.M.; Wain, R.L.; Fallis, A.G.; Jones,
     E.R.H.; Le Quan, M.; Page, C.B.; Thaller, V.; Shubrook, D.C.;
     Whitham, P.M. J. Chem. Soc. 1968, 2455-62.
50. Hargreaves, J.A.; Mansfield, J.W.; Coxon, D.T; Price, K.R.
     Phytochem. 1976, 15, 1119-21.
51. Hargreaves, J.A.; Mansfield, J.W.; Rossal, S. Physiol. Plant
     Path. 1977, 11, 227-42.
52. Letcher, R.M.; Widdowson, D.A.; Deverall, B.J.; Mansfield, J.W.
     Phytochem. 1970, 9, 249-52.
53. Downum, K.R.; Towers, G.H.N. J. Nat. Prod. 1983, 44, 98-103.
54. Downum, K.R.; Keil, D.J.; Rodriguez, E. Biochem. Syst. Ecol.
     1985, 13, 109-13.
55. DiCosmo, F.; Norton, R.; Towers, G.H.N. Naturwissenschaften
     1982, 69S, 550-51.
56. Garrod, B.; Lea, E.J.A.; Lewis, B.G. New Phytologist 1979, 83,
     463-71.
57. Schulte, K.E.; Wulfhorst, G. Archiv der Pharmacie 1977, 310,
     285-98.
58. Smith, D.A.; Banks, S.W. Phytochem. 1986, 25, 979-95.
59. Bakker, J.; Gommers, F.J.; Smits, L.; Fuchs, A.; de Vries, F.W.
     Photochem. Photobiol. 1983, 38, 323-29.
60. Sutton, D.C.; Gillan, F.T.; Susic, M. Phytochem. 1985, 24,
     2877-79.
61. Kemp, M.S.; Burden, R.S.; Loeffler, R.S.T. J. Chem. Soc.
     Perkin. Trans. I 1983, 2267-72.
62. Kemp, M.S.; Burden, R.S. J. Chem. Soc. Perkin Trans. I 1984,
     1441-43.
63. Burden, R.S.; Kemp, M.S.; Wiltshire, C.W. J. Chem. Soc. Perkin
     Trans. I 1984, 1445-48.

Chapter 22

Photodynamic Herbicides and Chlorophyll Biosynthesis Modulators

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Higher plants have been classified into four dif-
ferent greening groups depending on the monovinyl and
divinyl protochlorophyllide biosynthetic patterns at
night and in daylight. We have succeeded in demon-
strating that the photodynamic herbicidal suscepti-
bility of a particular plant species depends on its
greening group and on the chemical nature of the
δ-aminolevulinic acid (ALA)-dependent tetrapyrroles
that accumulate as a consequence of ALA-treatment.
Three groups of chemicals which modulate differen-
tially the monovinyl and divinyl monocarboxylic chlo-
rophyll biosynthetic routes have now been identified
namely (a) enhancers of ALA conversion to monovinyl
or divinyl tetrapyrroles, (b) inducers of ALA forma-
tion and conversion to monovinyl and divinyl tetra-
pyrroles and (c) inhibitors of divinyl tetrapyrrole
conversion to monovinyl tetrapyrroles. By combining
ALA with member(s) of one or more of the foregoing
groups of chlorophyll biosynthesis modulators, it has
become possible to design herbicidal formulations
which are very specific to certain crop and weed
plant species under a wide range of growth conditions.

In 1984, a novel approach for the design of useful herbicides was
reported (1). The concept and phenomenology were illustrated by
the description of an experimental herbicide based on a naturally
occurring amino acid, δ-aminolevulinic acid (ALA). Since then,
considerable progress has been achieved in expanding the scope of
this experimental herbicidal system, in understanding its mode of
action and in its development into a viable herbicide.

Review of the Experimental Photodynamic Herbicide System

Principles and Guidelines. The discovery of novel pesticides has
traditionally been the result of blind screening, that is the

result of experimentation involving trial and error. In a typical
year an agrichemical company may blind-screen 20,000 to 50,000
chemicals for herbicidal or insecticidal activity. The very few
chemicals that exhibit promise are then investigated further and
their efficacy, selectivity, environmental impact and phytotoxicity
are evaluated. In this undertaking, the understanding of the mode
of action of a particular pesticide is usually assigned a low
priority. Sometimes it is neither investigated nor understood.

      In 1982 we started a research effort aimed at the design of
novel herbicides by adopting an approach totally different from the
conventional industrial approach. The idea was to draw on basic
biological knowledge in order to design a herbicide which was based
on a preconceived mode of action.

Development of the Concept. The conceptual development of the
herbicide was naturally influenced by our past research experience
with the chemistry and biochemistry of the greening process. The
greening process is one of five major biological phenomena in the
biosphere. The other four being photosynthesis respiration, repro-
duction, and growth differentiation and development. It is most
obvious in the spring when deciduous annual and perennial plants
acquire their green color. This visual greening phenomenon is a
chemical expression of the biosynthesis and accumulation of chloro-
phyll (Chl) by developing chloroplasts. It is these green organ-
elles which are responsible for the conversion of solar energy to
chemical energy via the process of photosynthesis. Without the
normal occurrence of the greening process, photosynthesis is not
possible and organic life as we know it, is not possible either.

      Since the greening phenomenon occupies such a central position
in the economy of the biosphere, we reasoned that it should be
quite possible to design a herbicide with a mode of action rooted
into some facets of the greening process. This in turn raised the
important question of which aspects of the greening phenomenon
would best lend itself for such an undertaking. We first consid-
ered the possibility of designing a herbicide that may interfere
with the biosynthesis of Chl. Such a herbicide would act by pre-
venting the treated plants from replenishing the Chl of the fully
developed leaves and from forming new Chl to accommodate the expan-
sion of new leaves. We opted against this strategy because under
field conditions seedlings emerge from the soil essentially green
and their rate of Chl biosynthesis is as slow as their rate of Chl
turnover. In other words we conjectured that such a herbicide
would be a very slow acting herbicide, particularly on weeds that
had already attained a certain size.

      Another strategy offered more promise. We speculated that if
green plants could be induced to accumulate massive amounts of
tetrapyrroles, i.e. of Chl precursors, by spraying them with cer-
tain chemicals, there is a good chance that these compounds may be
developed into non-selective herbicides. We opted for this ap-
proach for several reasons. For one, tetrapyrroles and in particu-
lar Mg-tetrapyrroles, are notorious type II photosensitizers (1-3).
They have the tendency to absorb light energy and to photosensitize
the formation of singlet oxygen. The latter is a very powerful
oxidant and can trigger a free radical chain reaction that can

destroy biological membranes, nucleic acids, enzymes, and many
other proteins (3). Furthermore, metabolic Mg-tetrapyrroles are
extremely biodegradable (4-7) and their environmental impact would,
therefore, be negligible. What was not known however, was whether
a green plant that had acquired its full complement of Chl and
which was biosynthesizing Chl at rates commensurate with its slow
Chl turn-over rate, could be induced, by chemical treatment, to
accumulate enough tetrapyrroles to cause photodynamic damage. The
elucidation of this issue involved the determination of very small
amounts of tetrapyrroles in the presence of very large amounts of
Chl. Fortunately, this difficult analytical problem had been
tackled and solved about 10 years earlier (8-11). This in turn
made it possible to test and to demonstrate the most important
premise of the proposed herbicide concept, namely the possible
induction of Mg-tetrapyrrole accumulation and of photodynamic
damage in green plants by chemical treatment (1).

Choice of Herbicide. In view of the preconceived design, the
choice of herbicide became straightforward. For years it had been
known that dark-grown (i.e. etiolated) plants accumulated signifi-
cant amounts of tetrapyrroles upon treatment with ALA (12-14) (Fig.
1). This behaviour had its origin in three distinct phenomena:
(a) δ-aminolevulinic acid, a 5-carbon amino acid is the precursor
of heme and Chl in nature (15, 16), (b) the formation and availa-
bility of ALA for heme and Chl formation is highly regulated by
living cell, (17, 18) and (c) since etiolated plants contained only
small amounts of protochlorophylls (Pchls) [the immediate precur-
sors of chlorophyllide (Chl without phytol) and of Chl], but did
not contain any Chl (19), the Chl biosynthetic pathway in such
plants was extremely potent (20). It was poised for forming mas-
sive amounts of Chl, should the demand arise upon exposing the
plants to light (21). Upon treatment of such plants with ALA, an
important biosynthetic regulatory step was bypassed, namely the
regulation of ALA formation and availability to the plant (18).
Deluged with large amounts of ALA, the Chl biosynthetic machinery
of the etiolated plants was forced to convert the ALA to Mg-proto-
porphyrins and to Pchls in darkness and to convert some of the
latter to chlorophyllides and to Chl in the light (20-22). As a
consequence of the above considerations, and of the known photody-
namic effects of tetrapyrroles (vide supra), ALA appeared to be the
perfect candidate for a herbicide. Furthermore, since ALA was a
natural amino acid that occurred in all living cells and was an
integral part of the food chain, its environmental impact was
expected to be minimal. Therefore, what remained to be seen was
whether mature green plants would react to ALA treatment like
etiolated plants and accumulate enough tetrapyrroles to undergo
photodynamic damage. The demonstration of this phenomenon was
described in (1).

Discovery of the Selective Herbicidal Effect of ALA. As was just
mentioned, the ALA - based herbicide was meant to be a non-selec-
tive herbicide. Since it acted via the Chl biosynthetic pathway
and since the latter was such a fundamental process which was be-
lieved to be common to all green plants, we had no reason to
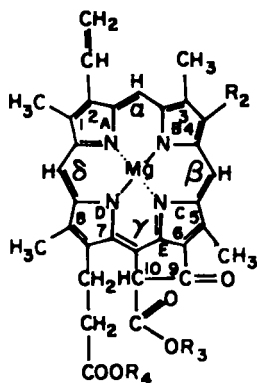
I PROTOCHLOROPHYLL (iDE)S

a. $R_2 = \_CH = CH_2$; $R_3 = \_CH_3$; $R_4 = $ F.Al; DV, 7_FAl.E, Pchl

b. $R_2 = \_CH_2\_CH_3$; $R_3 = \_CH_3$; $R_4 = $ F.Al; 2_MV, 7_FAl.E, Pchl

c. $R_2 = \_CH = CH_2$; $R_3 = \_CH_3$; $R_4 = $ H; DV, 7_COOH, 10_CO$_2$Me, Pchlide

d. $R_2 = \_CH = CH_2$; $R_3 = $ H; $R_4 = $ Alk; DV, 7_ Alk.E, 10_COOH Pchlide

e. $R_2 = \_CH_2\_CH_3$; $R_3 = \_CH_3$; $R_4 = $ H, 2_MV, 7_COOH, 10_CO$_2$ Me, Pchlide

f. $R_2 = \_CH_2\_CH_3$; $R_3 = $ H; $R_4 = $ Alk; 2_MV, 7_ Alk.E, 10_COOH Pchlide

Figure 1. Structure of monovinyl (MV) and divinyl (DV) Mg-
protoporphyrin monoester (MPE) and protochlorophyllide (Pchlide).
(Reproduced with permission from Reference 26. Copyright 1983
Nijhoff/Dr. W. Junk Publishers.)
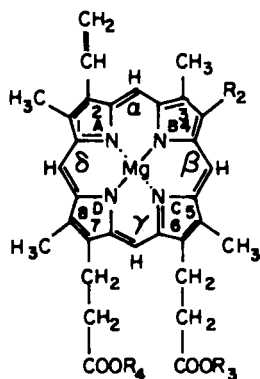
II. Mg PROTO diester, Mg PROTO monoester and Mg PROTO pools

a. $R_2 = -CH = CH_2$; $R_3 = -CH_3$; $R_4 = F.Al$; DV, 7_FAl.E, 6Me.P, Mg   Proto (DV Mg Proto diester)

b. $R_2 = -CH_2-CH_3$; $R_3 = -CH_3$; $R_4 = F.Al$; 2_MV, 7_FAl.E, 6Me.P Mg   Proto (MV Mg Proto dieste

c. $R_2 = -CH = CH_2$; $R_3 = -CH_3$; $R_4 = H$; DV, 7_COOH, 6Me.P, Mg Proto (DV Mg Proto 6 ME)

d. $R_2 = -CH = CH_2$; $R_3 = H$; $R_4 = Alk$; DV, 7_Alk.E, 6_COOH, Mg   Proto (DV Mg Proto 7 ester)

e. $R_2 = -CH_2-CH_3$; $R_3 = -CH_3$; $R_4 = H$, 2_MV, 7_COOH, 6Me.P, Mg Proto (MV Mg Proto 6 ME)

f. $R_2 = -CH_2-CH_3$; $R_3 = H$; $R_4 = Alk$. 2_MV, 7Alk.E, 6_COOH, Mg Proto (MV Mg Proto 7 ester)

g. $R_2 = -CH = CH_2$; $R_3 = H$; $R_4 = H$; DV Mg Proto

h. $R_2 = -CH_2-CH_2$; $R_3 = H$; = $R_4 = H$; 2_MV Mg Proto

Figure 1.--Continued. Structure of monovinyl (MV) and divinyl (DV) Mg-protoporphyrin monoester (MPE) and protochlorophyllide (Pchlide). (Reproduced with permission from Reference 26. Copyright 1983 Nijhoff/Dr. W. Junk Publishers.)

suspect even the possibility of an ALA herbicidal selectivity. It was, therefore, out of scientific routine that the herbicidal effect of ALA toward grassy monocotyledonous plants (monocots) such as corn, wheat, oat and barley was monitored. To our surprise the treated grassy monocots were essentially unaffected by the spray. This prompted us to expand the scope of the ALA-susceptibility studies to a variety of monocot and dicotyledonous (dicot) plants. Essentially, three types of herbicidal responses, named Type I, II and III, to ALA + 2,2'-dipyridyl (DPy), a Chl biosynthesis modulator, were noted (1): (a) type I response was exhibited by plants such as cucumber, which after treatment, died very rapidly, (b) type II response was exhibited by plants such as soybean which accumulated tetrapyrroles in the leafy tissues but not in the stems and cotyledons. Only the leaves exhibited photodynamic damage but the plants recovered and regrew vigorously, and (c) type III response was exhibited by monocots such as corn, wheat, oat and barley. Although the sprayed tissues did accumulate significant amounts of tetrapyrroles, photodynamic damage was minimal and the treated seedlings continued to grow and developed into healthy plants.

    Although at the time, we did not understand the biochemical origin of this differential response to the ALA treatment, we immediately realized the importance of this phenomenon, and we undertook the task of elucidating the molecular basis of this unexpected plant behaviour. This involved research dealing with the chemical and biochemical heterogeneity of the Chl biosynthetic pathway as well as research dealing with differences in the greening patterns of various higher plant species. The results of this research effort are described below.

The Multibranched Chl a Biosynthetic Pathway

On the basis of emerging experimental evidence, we had proposed in 1980, that the Chl biosynthetic pathway was not a single, linear chain of reactions that led to the formation of one Chl a and one Chl b chemical species as had been commonly believed (23-25). Instead we suggested that the experimental evidence was more compatible with the operation of a multibranched Chl biosynthetic pathway which led to the formation of several Chl a chemical species, having different functions in photosynthesis (25). This hypothesis was later on reinforced and expanded (18, 26). At that time we had no reason to suspect that various plant species may differ in their Chl biosynthetic activities until the differential ALA herbicidal response was observed. The latter could be readily explained on the basis of differences in the Chl biosynthetic pathways among various plant species. The investigation of this issue was therefore carried out within the conceptual framework of the multibranched Chl a biosynthetic pathway (26, 27), and led to the discovery of the 4 greening patterns of plants which are described below. The multibranched pathway reproduced in Fig. 2, consists of six parallel biosynthetic routes numbered 1 to 6. Most of the Chl in nature is actually formed via routes 2 and 5 which are the major monovinyl (MV) and divinyl (DV) monocarboxylic routes of that pathway. Monovinyl monocarboxylic tetrapyrroles possess

one vinyl and one free carboxylic group per macrocycle while DV
monocarboxylic tetrapyrroles possess two vinyl and one free car-
boxylic group per macrocycle (Fig. 1).   Some of the key observa-
tions that helped in the formulation of that pathway are described
in (28-39).

Classification of Higher Plants into Four Different Greening Groups

As we just mentioned (vide supra), there was no reason to believe
that green plants growing under natural field conditions, differed
in their Chl forming pathways.   Indeed, until very recently we
firmly believed that all green plants formed their Chl simultane-
ously via the six Chl biosynthetic routes depicted in Fig. 2.   This
notion came under question as a consequence of two observations:
(a) of the forementioned species-dependent differential ALA herbi-
cidal susceptibility which could be elegantly explained by the
occurrence of a differential Chl a biosynthetic heterogeneity in
various plant species and (b) of an earlier observation that etio-
lated (i.e. dark-grown) plant species did indeed differ in their
Pchlide and Chl biosynthetic capabilities during treatment with
alternating light/dark pulses (36).
     Before investigating the differential occurrence of various
Chl biosynthetic routes among different plant species, it was
mandatory, however, to develop the necessary analytical and pre-
paratory methodology.
     With the development of the appropriate experimental method-
ology (40, 41) it became possible to investigate the putative occur-
rence of a differential Chl biosynthetic heterogeneity in green
plants.   This was achieved by simply analyzing the MV and DV tetra-
pyrrole content of routes 1 and 6, routes 2 + 3 and routes 4 + 5
(Fig. 2) in various plant species growing under natural photoperi-
odic growth conditions.   It was considered that the amount of a
specific MV or DV tetrapyrrole belonging to a specific MV or DV
biosynthetic route and which was detectable at any particular time,
was related to the flow of tetrapyrrole intermediates via that
biosynthetic route at that particular time.   Because of the cyclic
alternation of night (darkness) and light (daylight) in nature, the
MV and DV tetrapyrrole content of the various plant species was
analyzed at two stages of the photoperiod: (a) at the end of the
dark phase of the photoperiod and (b) in the middle of the light
phase of the photoperiod.   The analysis at the end of the dark
phase of the photoperiod was meant to reflect the activity of the
biosynthetic routes at night, while analysis in the middle of the
day was meant to reflect the activity of the biosynthetic routes in
daylight.   It was conjectured that should differences be observed
among various plant species with respect to any two biosynthetic
routes, as for example between the MV and DV routes in darkness (D)
i.e. at night or in the light (L), i.e. in daylight, four meaning-
ful biosynthetic combinations may be observed, namely (a) dark
divinyl/light divinyl (DDV/LDV), (b) DMV/LDV, (c) DDV/LMV and (d)
DMV/LMV.   In the course of our investigations all four DV-MV
Pchlide combinations were observed (vide infra).
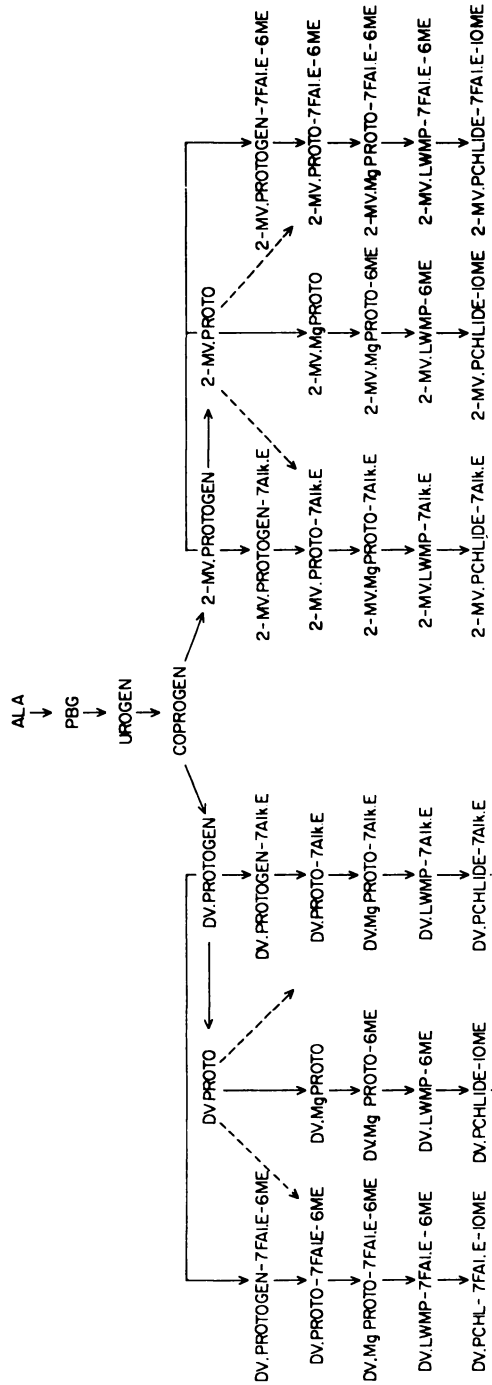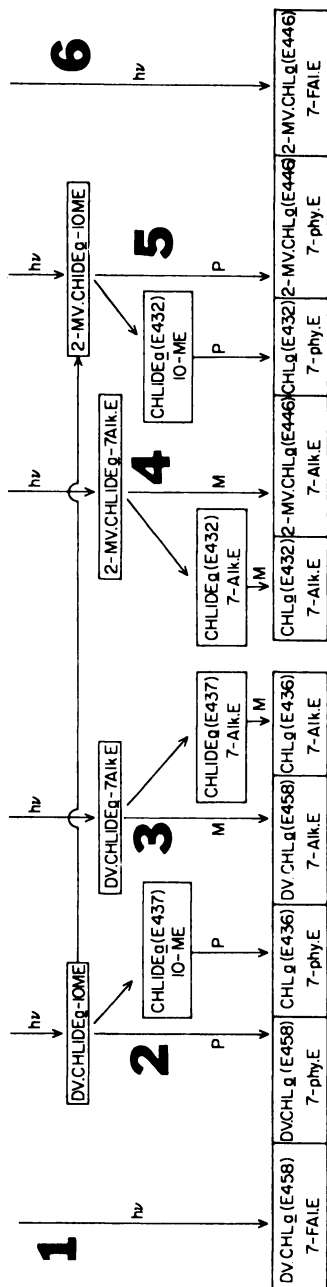
Figure 2. Six-branched Chla biosynthetic pathway: DV, divinyl; MV, monovinyl; FAl, fatty alcohol; Phy, phytol; E, ester; ME, methyl ester; Alk, alkyl group of unknown chain length; Me, methyl; ALA, δ-aminolevulinic acid; PBG, porphobillinogen; Urogen, uroporphyrinogen; Coprogen, coproporphyrinogen; Protogen, protoporphyrinogen; Proto, protoporphyrin IX; LWMP, longer wavelength metalloporphyrins (the putative intermediates of ring E formation); P, esterification with geranyl geraniol followed by stepwise conversion of the latter to phytol; M, methylation. (Reproduced with permission from Reference 26. Copyright 1983 Nijhoff/Dr. W. Junk Publishers.)

The DDV/LDV Greening Group. Plant species such as cucumber (Cucumis sativus L.), common purslane (Portulaca oleracea) and mustard (Brassica Juncea L. and Brassica kaber) belong in this group (38). During the dark phase of a 10 h dark/14 h light photoperiod, these plants accumulate mainly DV protochlorophyllide (Pchlide) and smaller amounts of MV Pchlide (42, 43). At daybreak, Chl formation proceeds via the DV-enriched Pchlide pool (42). Later on during the day, the proportion of MV Pchlide drops do a very low level and Chl formation proceeds mainly via the DV-enriched Pchlide pool (42).

The DMV/LDV Greening Group. This group appears to be the largest greening group of higher plants and include monocots, such as corn (Zea mays L.) wheat (Triticum secale L.) and barley (Hordeum vulgare) and dicots such as the common bean (Phaseolus vulgaris L.), soybean (Glycine max L.) and pigweed (Amaranthus retroflexus L.) (38). At the beginning of the dark phase of the photoperiod, these plants shift very rapidly from the DV Pchlide biosynthetic pattern (which prevails in daylight) to a MV Pchlide biosynthetic pattern. During the night they accumulate mainly MV Pchlide and very small amounts of DV Pchlide (42, 43). At daybreak, Chl formation proceeds via the MV enriched Pchlide pool. Under natural daylight, the plants shift back to a DV Pchlide accumulation pattern and form Chl mainly via the DV-enriched Pchlide pool (42, 43).

The DDV/LMV Greening Group. This recently discovered greening group was first described in 1986 (38) and (43) and so far includes fewer plant species than the other three greening groups. Its members include ginkgo (Ginkgo biloba) and violet species (Viola species). During the dark phase of the photoperiod, these plants accumulate mainly DV Pchlide and smaller amounts of MV Pchlide. At daybreak, they form Chl mainly via the DV-enriched Pchlide pool and later on in daylight form Chl via the MV-enriched Pchlide pool.

The DMV/LMV Greening Group. Likewise this greening group was also recently described (38, 43). It includes plant species such as apple (Pyrus malus) and Johnson grass (Sorghum halepense). During the dark phase of the photoperiod these plants accumulate predominantly MV Pchlide and smaller amounts of DV Pchlide. At daybreak and later on during daylight they form Chl mainly via the MV-enriched Pchlide pool (38, 43).

Molecular Origin of the Various Greening Patterns in Higher Plants

Since we strongly suspected that the differential ALA-dependent photodynamic susceptibility of green plants was closely tied to the biochemical origin of the differential greening patterns of higher plants, this relationship was next investigated.

Biosynthetic Origin of the DV and MV Pchlide Accumulation Patterns in the DDV/LDV Greening Group of Plants. The origin of the DV Pchlide accumulation pattern in this greening group was readily demonstrated with the use of the DDV/LDV cucumber cell-free system

described in (41). It was shown to originate in biosynthetic routes 2 + 3 (Fig. 2). This was achieved by demonstrating the conversion of ALA to DV Pchlide via DV protoporphyrin (Proto), DV Mg-Proto and DV Mg-Proto monoester in darkness (38, 39).

A more important issue, however, was whether the MV Pchlide accumulation pattern originated in the conversion of DV Pchlide to MV Pchlide or whether it originated somewhere else. This question was settled by demonstrating the biosynthesis of MV Pchlide via the MV monocarboxylic routes 4 + 5 (Fig. 2). It was shown that although the cucumber etiochloroplasts system was not capable of converting DV Pchlide to MV Pchlide, it did convert, very efficiently ALA to MV Pchlide via MV Proto, MV Mg-Proto and MV Mg-Proto monoester (38, 39). Furthermore, it was shown that in this system, DV routes 2 + 3 and MV routes 4 + 5 (Fig. 2) were (a) either not interconnected, i.e. at the level of DV Pchlide, or (b) were very weakly interconnected at site(s) between DV Proto and DV Pchlide (38, 39).

The regulation of the DV and MV monocarboxylic routes in this greening group of plants is presently under investigation.

Biosynthetic Origin of the DV and MV Pchlide Accumulation Patterns in the DMV/LDV Greening Group. The origin of the DV Pchlide accumulation pattern in this greening group was investigated with the DMV/LDV barley cell-free system described in (41). The origin of the DV Pchlide accumulation pattern was shown to reside in biosynthetic routes 2 + 3 (Fig. 2) by demonstrating the conversion of ALA to DV Pchlide via DV Proto, DV Mg-Proto and DV Mg-Proto monoester (38, 39).

The origin of the MV Pchlide accumulation pattern was however considerably more complex than in DDV/LDV plants. About 30% of the MV Pchlide appeared to be formed from ALA via the MV monocarboxylic routes, i.e. routes 4 + 5 (Fig. 2). This was evidenced by the dark-conversion of ALA to MV Pchlide via MV Proto, MV Mg-Proto and MV Mg-Proto monoester in barley etiochloroplasts poised in the MV Pchlide accumulation mode (38, 39). A sizable fraction of the MV Pchlide pool appeared to be also formed from DV Proto, DV Mg Proto and DV Mg Proto monoester but not from DV Pchlide (38, 39). This was apparently accomplished via one or more DV tetrapyrrole reductase(s) that converted DV tetrapyrroles to MV tetrapyrroles by reduction of the vinyl group at position 4 of the macrocycle to an ethyl group (Fig. 1). As a consequence, in this greening group of plants, the DV and MV monocarboxylic biosynthetic routes were very strongly interconnected (38, 39). The precise number and biochemical site(s) of the DV tetrapyrrole reductases is presently under investigation. Very recent data also indicates that in DMV/LDV plants, under certain greening conditions, a small fraction of the DV Pchlide pool may be convertible to MV Pchlide via a DV Pchlide reductase (B. C. Tripathy and C. A. Rebeiz, unpublished).

Investigation of the regulation of the MV and DV monocarboxylic routes in DMV/LDV plants is in progress. Likewise, the biosynthetic origin of the DV and MV Pchlide accumulation patterns in the other two greening groups of plants, i.e. in the DDV/LMV and the DMV/LMV group is also under investigation.

Biochemical Origin of the Differential ALA-dependent Photodynamic Susceptibility of Green Plants

The molecular basis of the differential photodynamic susceptibility of various plant tissues and plant species to ALA treatment was investigated within the framework of the following hypothesis: (a) that the accumulation of tetrapyrroles by ALA-treated tissues was a necessary but not a sufficient condition for the occurrence of photodynamic damage and (b) that in the event of tetrapyrrole accumulation the occurrence and extent of photodynamic damage was likely to depend (α) on the extent of tetrapyrrole accumulation, (β) on the greening group of the treated plant and (γ) on the chemical nature of the accumulated tetrapyrrole. It was also recognized that the extent of a plant species photodynamic susceptibility to ALA treatment may be due to one or more of the forementioned conditions. The logistics behind the above hypothesis was based upon the following observations.

The proposed necessity of tetrapyrrole accumulation for the occurrence of photodynamic damage is a consequence of the basic mode of action of ALA toward susceptible plant species. Indeed the relationship between ALA treatment, total tetrapyrrole accumulation and photodynamic damage has already been demonstrated with susceptible plant species such as cucumber (1). On the other hand, the proposal of the "non-sufficiency" condition was on the basis that although some treated plant species accumulated large amounts of tetrapyrroles, they did not undergo significant photodynamic damage (1).

In susceptible plants that responded to ALA treatment by accumulating tetrapyrroles, the proposed dependence of photodynamic damage on the extent of tetrapyrrole accumulation is again an obvious consequence of the demonstrated dependence of photodynamic damage on total tetrapyrrole accumulation (1).

Finally in plant species capable of ALA-dependent tetrapyrrole accumulation, the proposed dependence of photodynamic damage upon the greening group of the treated plant as well as upon the chemical nature of the accumulated tetrapyrrole was based on experimental evidence that will be described below.

Dependence of Photodynamic Damage on the Extent of Tetrapyrrole Accumulation: Case Study of the Differential Photodynamic Susceptibility of Soybean Cotyledons and Primary Leaves to ALA Treatment.

This case study explores the molecular basis of the differential photodynamic susceptibility of soybean cotyledons and soybean primary leaves to ALA-treatment. As may be recalled, although the primary leaves of soybean seedlings are very susceptible to ALA treatment, soybean stems and cotyledons are not (1). As a consequence, although the primary leaves of ALA-treated seedlings die within a few hours of exposure to daylight, the intact stems and cotyledons sustain the production of new leaves and the treated seedlings soon recover.

The resistance of soybean stems to ALA treatment is obviously related to the lack of tetrapyrrole accumulation by the treated stems as described in (1). In order to determine whether the response of soybean cotyledons, a DMV/LDV tissue (42), to ALA

treatment was also rooted in a lack of tetrapyrrole accumulation,
the following experiment was performed. Greenhouse grown soybean
seedlings were treated with a 5 mM ALA + 15 mM DPy solution pre-
cisely as described in (1). After wrapping the plants in aluminum
foil and dark incubation for 17 h (1), tetrapyrrole accumulation by
the primary leaves and by the cotyledons was determined and the
seedlings were exposed to daylight in the greenhouse to induce
photodynamic damage. In this experiment total ALA-dependent tetra-
pyrrole accumulation by the primary leaves amounted to 201 nmoles
per 100 mg of tissue protein while the cotyledons accumulated only
11 nmoles of tetrapyrroles per 100 mg protein. After a few hours
in daylight, photodynamic damage to the leaves amounted to 100%
while the cotyledons were unaffected.

     Altogether, these results indicated that the lack of photo-
dynamic damage to soybean cotyledons was due to poor exogenous
ALA-dependent tetrapyrrole accumulation by this tissue.

Dependence of Photodynamic Damage on the Chemical Nature of the
Accumulated Tetrapyrrole and on the Greening Group of the Treated
Plants. In these preliminary studies, only three model plant
systems have been used: (a) cucumber seedlings, in the cotyledon
stage as a representative of the DDV/LDV group of plants and (b)
corn, and to a lesser extent soybean seedlings, as monocot and
dicot representatives of the DMV/LDV greening group. The tentative
conclusions drawn from these studies are, therefore, limited in
scope and in the future may have to be adjusted to accommodate
additional observations derived from ALA-susceptibility studies
with DDV/LMV, DMV/LMV as well as from additional DDV/LDV and
DMV/LDV plant species.

     In order to correlate the accumulation of specific tetra-
pyrroles with induction of photodynamic damage, we have used a
group of 13 chemicals which act in concert with ALA. The mode of
action of these chemicals, which will be referred to as "modula-
tors" of Chl biosynthesis, will be discussed in some details later
on (vide infra). They were a convenient tool in demonstrating
relationships between the accumulation of specific tetrapyrroles
and photodynamic damage. Indeed, when used in concert with ALA,
they resulted in the preponderant accumulation of specific MV or DV
tetrapyrroles as described below.

     In these experiments we used low concentrations of ALA (5 mM)
in conjunction with higher concentrations (10 to 30 mM) of each one
of the 13 Chl biosynthesis modulators. The idea was to induce only
limited photodynamic damage in order to correlate more precisely
between the extent of the latter and the accumulation of specific
tetrapyrroles. The results of these experiments are summarized
below.

Case Study 1: Induction of Photodynamic Damage by ALA-dependent
Accumulation of MV Pchlide in Cucumber, a DDV/LDV Plant Species but
not in Corn a DDMV/LDV Plant Species. In seven of the thirteen
different treatments which used ALA in conjunction with increasing
concentrations of individual members of the 13 Chl biosynthesis
modulators, MV Pchlide was the preponderant tetrapyrrole that
accumulated in the dark in the treated cucumber seedlings. In

every one of those 7 treatments the best correlation was observed
between MV Pchlide dark-accumulation and photodynamic damage. One
such experiment is described in Table IA. These results indicated
that cucumber a DDV/LDV plant species was photodynamically suscepti-
ble to ALA-dependent dark accumulation of MV Pchlide.

In order to determine whether this conclusion was also valid
for DMV/LDV plant species, similar experiments were performed on
corn. In all these experiments, the ALA-dependent dark-accumula-
tion of MV Pchlide resulted either in the absence of photodynamic
damage, as described in Table IB or in very minor damage from which
the seedlings recovered very rapidly as reported in (1).

As a consequence of these results we propose that while
DDV/LDV plant species such as cucumber are photodynamically sus-
ceptible to ALA-dependent MV Pchlide dark accumulation, DMV/LDV
plant species such as corn are either not susceptible or much less
photodynamically susceptible than the DDV/LDV plant species. This
hypothesis is presently being tested on a larger number of plant
species belonging to the two forementioned greening groups.

Case Study 2: Induction of ALA-dependent DV Pchlide Dark Accumula-
tion Cause Less Photodynamic Damage in Cucumber a DDV/LDV Plant
Species than in Soybean, a DMV/LDV Plant Species. Four of the
thirteen Chl biosynthesis modulators resulted in the dark-accumula-
tion of more DV Pchlide than MV Pchlide in particular at the higher
concentration range (20 and 30 mM) of the modulators. In these
experiments, although the incidence of photodynamic damage did
correlate with ALA-induced DV Pchlide dark accumulation (Table IC),
the extent of photodynamic damage was usually less pronounced than
when cucumber was forced to accumulate MV Pchlide (Table II). It
is not known at this stage whether the reduced photodynamic damage
induced by modulators that cause the preponderant dark-accumulation
of DV Pchlide in cucumber is due to a lower photodynamic suscepti-
bility of DDV/LDV plant species per unit of accumulated DV Pchlide
or to some other causes.

In order to determine whether DMV/LDV plant species exhibit a
higher photodynamic susceptibility to DV Pchlide dark-accumulation
than DDV/LDV plant species, similar experiments are now being
performed on soybean seedlings in the primary leaf stage. Pre-
liminary results have so far indicated that primary leaves of
soybean are extremely susceptible to ALA-based treatments that do
result in DV Pchlide accumulation in cucumber. At this stage we
have no reason to doubt the correlation of this photodynamic sus-
ceptibility with DV Pchlide accumulation by the soybean primary
leaves.

Case Study 3: Induction of Photodynamic Damage by ALA-Dependent DV
Mg-Protoporphyrin (Monoester) Accumulation in Both Cucumber and
Corn. Two of the 13 Chl biosynthesis modulators caused the massive
ALA-dependent accumulation of DV Mg-protoporphyrin (monoester)
[MP(E)] in cucumber, a DDV/LDV plant species, and in corn, a
DMV/LDV plant species. In both species it is the accumulation of
DV MP(E) that exhibited the best correlation with photodynamic
damage (Table I, D, E). Corn, however, recovered after a few days

Table I. Differences in ALA-Dependent Tetrapyrrole Accumulation and Photodynamic Damage Between DDV/LDV and DMV/LDV Plant Species

Seedlings were sprayed in the late afternoon with solvent only or with solvent containing 5 mM ALA (130 g/acre) and a modulator (10 to 30 mM) at a rate of 40 gallons per acre: The solvent consisted of acetone: ethyl acetate: tween 80: H$_2$O (45:45:1:90 v/v/v/v). The sprayed plants were wrapped in aluminum foil and placed in darkness at 28°C for about 17 h. The next morning, the treated plants were sampled for tetrapyrrole analysis then were exposed to daylight in the greenhouse for the evaluation of photodynamic damage. For more details consult (1). Pchlide = protochlorophyllide; MP(E) = Mg proto + Mg proto monoester; MV = monovinyl; DV = divinyl; n.s. = not significant. Adapted from (27).

Experiment	Plant Species	Treatment	Photodynamic Damage	Pchlide		MP(E)	
			(%)	MV	DV	MV	DV
				Exogenous ALA-dependent tetrapyrrole accumulation[a] in nmoles per 100 mg proteins			
A	Cucumber	Solvent only	0	0.00	0.00	0.00	0.00
		5 mM ALA	43	51.38	17.81	0.38	-0.60
		10 mM 2-pyridine aldoxime	0	4.55	3.08	0.13	-0.84
		5 mM ALA + 10 mM 2-pyridine aldoxime	83	44.64	4.96	0.77	-0.52
		20 mM 2-pyridine aldoxime	0	12.16	1.94	-0.50b	-0.34
		5 mM ALA + 20 mM 2-pyridine aldoxime	83	35.69	8.12	0.44	0.08
		30 mM 2-pyridine aldoxime	0	17.60	3.34	0.16	0.43
		5 mM ALA + 30 mM 2-pyridine aldoxime	68	65.08	17.62	0.32	-0.20
		Correlation coefficient		0.817	0.569		
		Level of significance[c]		5%	n.s.		

Table I. Continued. Differences in ALA-Dependent Tetrapyrrole Accumulation and Photodynamic Damage Between DDV/LDV and DMV/LDV Plant Species

Experiment	Plant Species	Treatment	Photodynamic Damage (%)	Exogenous ALA-dependent tetrapyrrole accumulation[a] in nmoles per 100 mg proteins			
				Pchlide		MP(E)	
				MV	DV	MV	DV
B	Corn	Solvent only	0	0.00	0.00	0.00	0.00
		5 mM ALA	0	17.36	-2.30	-5.07	-1.13
		10 mM picolinic acid	0	39.86	-2.73	-0.06	0.85
		5 mM ALA + 10 mM picolinic acid	0	16.03	-1.83	-1.13	1.64
		20 mM picolinic acid	0	49.93	15.79	8.14	8.12
		5 mM ALA + 20 mM picolinic acid	0	10.24	-1.69	5.07	17.01
		30 mM picolinic acid	0	58.49	0.20	2.52	20.24
		5 mM ALA + 30 MM picolinic acid	0	81.29	4.10	0.04	20.67
		Correlation coefficient		0.000	0.000	0.000	0.000
		Level of significance		n.s.	n.s.	n.s.	n.s.

C Cucumber	Solvent only	0	0.00	0.00	0.00	0.00
	5 mM ALA	55	30.62	6.41	0.33	0.18
	10 mM 1,7-phenanthroline	5	10.50	6.94	1.44	-0.58
	5 mM ALA + 10 mM 1,7-phenanthroline	45	11.07	8.45	0.45	-0.22
	20 mM 1,7-phenanthroline	33	1.49	4.26	-0.34	1.48
	5 mM ALA + 20 mM 1,7-phenanthroline	50	7.32	9.37	-0.16	0.41
	30 mM 1,7-phenanthroline	43	2.52	8.72	0.36	0.13
	5 mM ALA + 30 mM 1,7-phenanthroline	63	1.73	15.30	1.88	0.92
	Correlation coefficient		0.29	0.753		
	Level of significance		n.s.	5%		
D Cucumber	Solvent only	0	0.00	0.00	0.00	0.00
	5 mM ALA	10	15.48	3.15	-0.04	0.18
	10 mM 1,10-phenanthroline	73	25.25	9.09	4.44	48.47
	5 mM ALA + 10 mM 1,10-phenanthroline	90	34.35	23.38	-0.11	75.20
	20 mM 1,10-phenanthroline	93	12.93	8.78	-0.11	56.77
	5 mM ALA + 20 mM 1,10-phenanthroline	93	33.16	32.69	1.12	85.38
	30 mM 1,10-phenanthroline	100	10.20	7.92	0.95	45.92
	5 mM ALA + 30 mM 1,10-phenanthroline	100	8.08	14.12	6.03	44.43
	Correlation coefficient		0.413	0.623	0.364	0.861
	Level of significance		n.s.	20%	n.s.	1%
	Solvent only	0	0.00	0.00	0.00	0.00
	5 mM ALA	0	12.20	-0.27	9.52	-2.60
	10 mM 2,2'-dipyridyl	0	40.45	-0.41	1.53	-1.85
	5 mM ALA + 10 mM 2,2'-dipyridyl	30^d	98.32	8.52	20.01	39.04

Table I. Continued. Differences in ALA-Dependent Tetrapyrrole Accumulation and Photodynamic Damage Between DDV/LDV and DMV/LDV Plant Species

Experiment	Plant Species	Treatment	Photodynamic Damage (%)	Pchlide [a] (MV)	Pchlide (DV)	MP(E) (MV)	MP(E) (DV)
				Exogenous ALA-dependent tetrapyrrole accumulation[a] in nmoles per 100 mg proteins			
E	Corn	20 mM 2,2'-dipyridyl	0	11.01	1.01	4.79	1.60
		5 mM ALA + 2,2'-dipyridyl	31	34.70	-0.65	5.12	7.33
		30 mM 2,2'-dipyridyl	40	104.28	-1.18	7.26	9.41
		5 mM ALA + 30 mM 2,2'-dipyridyl	80	12.87	-0.39	13.54	25.13
		Correlation coefficient		0.377	0.068	0.557	0.700
		Level of significance		n.s.	n.s.	n.s.	10%

[a] Is the difference between the tetrapyrrole content of the ALA or ALA + modulator-treated plants and that of the control plants which were sprayed with solvent only.

[b] Negative values indicate a drop in content in comparison to the content of the control plants.

[c] Refers to the probability that for a population for which the correlation coefficient (r) is equal to zero, a sample of size n can be taken, for which the correlation equals or exceeds the calculated value of r which is reported in the table for a given sample.

[d] Since corn plants recovered from photodynamic damage, the values reported for corn were determined two days after spraying. Those reported for cucumber were determined 10 days after spraying.

Table II.   Response of Cucumber, a DDV/LDV Plant Species and of Soybean, a DMV/LDV Plant Species to Enhancers of ALA Conversion to MV Protochlorophyllide

Treatment conditions, abbreviations and definitions are as in Table I.   Adapted from (27).

Chlorophyll biosynthesis modulator	Treatment	Photodynamic damage (%)		Cucumber			
				Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)			
				Pchlide		MPE	
		Soybean	Cucumber	MV	DV	MV	DV
	Solvent only	0	0	0.00	0.00	0.00	0.00
	5 mM ALA	0	40	9.69	4.62	0.83	-0.67
	10 mM modulator	0	0	3.99	3.13	0.97	-0.90
	5 mM ALA + 10 mM modulator	0	43	21.24	15.34	1.70	-0.60
	20 mM modulator	0	0	4.27	3.48	1.36	-1.19
2-pyridine aldehyde	5 mM ALA + 20 mM modulator	0	60	22.33	15.73	0.87	-0.98
	30 mM modulator	0	0	3.49	3.67	0.88	0.06
	5 mM ALA + 30 mM modulator	0	70	32.89	21.96	0.43	1.30
	Correlation coefficient			0.945	0.907	-	-
	Level of significance			0.1%	1%	-	-

Table II. Continued. Response of Cucumber, a DDV/LDV Plant Species, and of Soybean, a DMV/LDV Plant Species, to Enhancers of ALA Conversion to MV Protochlorophyllide

Chlorophyll biosynthesis modulator	Treatment	Photodynamic damage (%)		Cucumber			
				Pchlide		MPE	
		Soybean	Cucumber	MV	DV	MV	DV
				Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)			
picolinic acid	Solvent only	0	0	0.00	0.00	0.00	0.00
	5 mM ALA	3	34	12.05	4.03	-0.15	-0.51
	10 mM modulator	3	0	2.92	4.20	-0.18	-0.69
	5 mM ALA + 10 mM modulator	6	71	16.87	12.65	1.76	-0.59
	20 mM modulator	3	0	11.08	6.20	-0.33	0.70

5 mM ALA + 20 mM modulator	12	80	39.87	17.35	-0.87	0.55
30 mM modulator	6	10	2.68	2.35	-0.72	0.69
5 mM ALA + 30 mM modulator	21	90	21.47	9.06	-0.92	3.99
Correlation coefficient			0.828	0.811	-	-
Level of significance			5%	5%	-	-
Solvent only	0	0	0.00	0.00	0.00	0.00
5 mM ALA	12	80	11.94	0.19	0.42	2.06
10 mM modulator	8	0	-2.94	-0.96	0.20	0.27
5 mM ALA + 10 mM modulator	16	70	18.08	1.96	-1.01	0.92
20 mM modulator	17	0	-2.73	0.31	0.18	0.47
5 mM ALA + 20 mM modulator	19	70	17.32	3.70	-0.43	-0.16
30 mM modulator	15	0	6.14	1.89	0.75	0.28
5 mM ALA + 30 mM modulator	21	73	41.80	4.87	1.07	-0.01
Correlation coefficient			0.779	0.598	-	-
Level of significance			5%	20%	-	-

2,2'-dipyridyl disulfide

of growth. These results indicated that the accumulation of DV
MP(E) by a plant was likely to cause photodynamic damage, irrespec-
tive of the greening group to which the plant belonged.

Origin of the Differential Photodynamic Susceptibility of Various
Plant Species to ALA-Dependent Tetrapyrrole Accumulation: A
Working Hypothesis. On the basis of the above, albeit limited,
observations we now propose the following working hypothesis: (a)
that Pchlide is the most ubiquitous of the damage-causing photo-
dynamic tetrapyrroles that accumulate as a consequence of ALA-based
treatments, (b) that DDV/LDV plant species are likely to be more
photodynamically susceptible to ALA-based dark treatments that lead
to MV Pchlide accumulation, than to those that lead to DV Pchlide
accumulation. However, it remains to be determined whether this is
due to differences in the photodynamic damage-causing potential
between equimolar amounts of MV and DV Pchlide or whether it is due
to other factors, (c) that DMV/LDV plant species are photodynami-
cally more susceptible to DV Pchlide than to MV Pchlide accumula-
tion, and (d) that both DDV/LDV and DMV/LDV plant species are
highly susceptible to DV MP(E) accumulation.
     As was already pointed out, the premises of this hypothesis
are likely to be expanded and/or refined in order to accommodate
additional observations derived from additional photodynamic sus-
ceptibility studies of the four greening groups of plants. Fur-
thermore, it would be very desirable to determine the reason why
DDV/LDV plant species appear to be more susceptible to ALA-depen-
dent MV Pchlide dark-accumulation while DMV/LDV plant species
appear to be more susceptible to DV tetrapyrrole dark-accumulation.

Modulation of AlA-dependent Tetrapyrrole Accumulation and
Concommitant Modulation of Photodynamic Damage by Chlorophyll
Biosynthesis Modulators

The observation that the photodynamic susceptibility of a plant
species depended on the greening group of the particular plant
species as well as on the nature of the accumulated tetrapyrroles
had obvious biotechnological implications. It suggested that
chemicals that may be able to induce ALA-treated plants, belonging
to a certain greening group, to accumulate the "wrong" type of MV
or DV tetrapyrrole, while inducing other plant species, belonging
to other greening groups, to accumulate the "right" type of MV or
DV tetrapyrrole may act as photodynamic herbicide modulators. In
other words, such chemicals when used in conjunction with ALA had
the potential to expand the ALA herbicide into a highly selective
system of photodynamic herbicides.
     With this in mind we undertook a literature search for chemi-
cals and biochemicals known to affect in a general way, Chl and
Pchl formation (44-46). We then determined the specific effect of
these chemicals on the various Chl a biosynthetic routes described
in Fig. 2.
     This research effort resulted in the identification of a total
of 13 chemicals which acted in concert with ALA and which were
capable of modulating the Chl a biosynthetic pathway. These chemi-
cals were, therefore, designated collectively as Chl a biosynthesis

modulators. They were classified into three major groups depending
on their mode of action. One group consisted of enhancers of ALA
conversion to tetrapyrroles. Another group consisted of inducers
of ALA biosynthesis and of tetrapyrrole accumulation while a third
group consisted of inhibitors of MV protochlorophyllide
accumulation.

The effect of these various groups of Chl a biosynthesis
modulators on the Chl a biosynthetic pathway and on induced photo-
dynamic damage is described below.

Enhancers of ALA Conversion to Tetrapyrroles. To qualify as an
enhancer of ALA conversion to a particular MV or DV tetrapyrrole it
was considered that: (a) a particular Chl biosynthesis modulator
should not result in a significant accumulation of the MV or DV
tetrapyrrole in question, when applied to a plant in the absence of
exogenous ALA, but (b) at certain concentrations of the modulator,
when the latter is used jointly with exogenous ALA, it should
enhance the dark tetrapyrrole conversion of exogenous ALA, into
that particular MV or DV tetrapyrrole, over and beyond the ALA
control. A significant accumulation of a particular tetrapyrrole
was in turn defined arbitrarily as an amount of that tetrapyrrole
that approached or exceeded the net dark-conversion rate of a 5 mM
exogenous ALA treatment into that tetrapyrrole.

Enhancers of ALA conversion to tetrapyrroles were observed to
fall into two distinct groups namely (a) enhancers of ALA conver-
sion to MV Pchlide and (b) enhancers of ALA conversion to DV
Pchlide. These two subgroups of enhancers will now be discussed
separately.

Enhancers of ALA Conversion to MV Pchlide. 2-Pyridine aldehyde,
picolinic acid, 2,2'-dipyridyl disulfide (Table II) and 2-pyridine
aldoxime, the latter in the higher concentration range (Table I, A)
were found to enhance preferentially the dark-conversion of exo-
genous ALA to MV Pchlide in DDV/LDV plant species such as cucumber.
It should be emphasized, however, that although these compounds
enhanced preferentially the dark-conversion of ALA to MV Pchlide,
some of them also enhanced significantly, but to a lesser extent,
the dark-conversion of exogenous ALA to DV Pchlide.

In DDV/LDV plant species such as cucumber, a higher correla-
tion was observed between photodynamic damage and the dark-accumula-
tion of MV Pchlide, than between photodynamic damage and the accumu-
lation of DV Pchlide. No significant accumulation of either MV or
DV Mg-protoporphyrins was observed (Table II).

Treatment of soybean with these same enhancers of exogenous
ALA conversion to MV Pchlide resulted in minimal or no photodynamic
damage (Table II). This is fully compatible with the proposed
differential susceptibility hypothesis. Particularly if soybean, a
DMV/LDV plant species reacted to treatment with ALA and 2-pyridine
aldehyde, picolinic acid, 2,2'-dipyridyl disulfide or 2-pyridine
aldoxime, as did cucumber a DDV/LDV plant species, by accumulating
MV Pchlide. This question is presently under investigation.

Enhancers of ALA Conversion to DV Pchlide. 4,4'-dipyridyl, 2,2'-
dipyridyl amine and phenanthridine were observed to fall into this

group of Chl biosynthesis modulators. At the higher-concentration
range, they enhanced preferentially the dark conversion of exo-
genous ALA to DV Pchlide in treated cucumber seedlings. Since
cucumber is a DDV/LDV plant species, it was less photodynamically
sensitive to fluctuations in its DV Pchlide than to fluctuations in
its MV Pchlide content. This in turn was reflected by a better
correlation between photodynamic damage and MV Pchlide accumulation
than between photodynamic damage and DV Pchlide accumulation (Table
III). No significant accumulation of either MV or DV Mg-protopor-
phyrins was observed.

     In contrast to cucumber, dark-treatment of soybean with ALA
together with the forementioned enhancers of ALA conversion to DV
Pchlide, resulted in extensive photodynamic damage (Table III).
This was the expected phenomenology if the dark treatment of soy-
bean, a DMV/LDV plant species, with ALA and 4,4'-dipyridyl, 2,2'-
dipyridyl amine or phenanthridine had triggered an enhancement or
an induction of DV tetrapyrrole accumulation. This matter is
presently under investigation.

Inducers of Tetrapyrrole Accumulation. To qualify as an inducer of
tetrapyrrole accumulation, it was considered that a particular Chl
biosynthesis modulator should, at certain concentrations, result in
a significant accumulation of a particular MV or DV tetrapyrrole,
when applied to a plant in the absence of exogenous ALA. Here
again, significant accumulation of a particular tetrapyrrole was
arbitrarily defined as an amount of that tetrapyrrole that ap-
proaches or exceeds the net dark-conversion rate of a 5 mM exogen-
ous ALA treatment into that tetrapyrrole. Furthermore, at certain
concentrations of the inducer, the latter, in combination with ALA,
should result in the accumulation of higher levels of the particu-
lar MV or DV tetrapyrrole than when ALA or the inducer are applied
to the plant separately.

     1,10-phenanthroline (i.e. 0-phenanthroline) (Table I, D) and
2,2'-dipyridyl (Table IV) were observed to act preferentially as
inducers of DV Mg-protoporphyrin + DV Mg-protoporphyrin monoester
[DV MP(E)] accumulation. It should be noted that while 1,10-phenan-
throline preferentially induced the biosynthesis and accumulation
of DV MP(E), it also induced, to a lesser extent, the accumulation
of DV Pchlide (Table I D). 2,2'-dipyridyl (Table IV) did not
exhibit this property. In cucumber the highest correlation was
observed between DV MP(E) accumulation and photodynamic damage
(Tables I, D and IV).

     Soybean a DMV/LDV plant species was equally susceptible to
treatment with 2,2'-dipyridyl (Table IV) and to 1,10-phenanthroline
(data not shown). This in turn was compatible with the proposed
mode of action hypothesis. Investigations of the quantitative re-
lationships between the induction of specific tetrapyrrole accumula-
tion and the incidence of photodynamic damage in DMV/LDV plant
species such as soybean are in progress.

Inhibitors of MV Protochlorophyllide Accumulation. To qualify as
an inhibitor of MV Pchlide accumulation, it was considered that a
particular Chl biosynthesis modulator (a) when used alone, should
result in the inhibition of MV Pchlide accumulation, in comparison

Table III.   Response of Cucumber, a DDV/LDV Plant Species and of Soybean, a DMV/LDV Plant Species to Enhancers of ALA Conversion to DV Protochlorophyllide. Adapted from (27).

Treatment abbreviations and definitions are as in Table I.

Chlorophyll biosynthesis modulator	Treatment	Photodynamic damage (%)		Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein) Cucumber			
				Pchlide		MPE	
		Soybean	Cucumber	MV	DV	MV	DV
	Solvent only	0	0	0.00	0.00	0.00	0.00
	5 mM ALA	10	20	19.66	4.06	-1.09	-0.82
	10 mM modulator	15	0	-1.82	-1.90	-1.09	-1.02
	5 mM ALA + 10 mM modulator	50	15	11.09	4.44	-0.31	-0.69
4,4'-Dipyridyl	20 mM modulator	58	0	-1.20	-0.44	-0.56	-0.85

Table III. Continued. Response of Cucumber, a DDV/LDV Plant Species, and of Soybean, a DMV/LDV Plant Species, to Enhancers of ALA Conversion to DV Protochlorophyllide

Chlorophyll biosynthesis modulator Treatment	Photodynamic damage (%)		Cucumber			
			Pchlide		MPE	
				Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)		
	Soybean	Cucumber	MV	DV	MV	DV
5 mM ALA + 20 mM modulator	76	75	20.69	22.68	0.31	-0.09
30 mM modulator	85	0	-1.34	9.21	0.73	-0.81
5 mM ALA + 30 mM modulator	93	25	22.56	34.54	-0.08	-0.39
Correlation coefficient	-	-	0.760	0.647	-	-
Level of significance	-	-	5%	10%	-	-
Solvent only	0	0	0.00	0.00	0.00	0.00
5 mM ALA	15	25	16.23	3.87	-0.69	-0.87
10 mM modulator	0	-	-	-	-	-
5 mM ALA + 10 mM modulator	36	25	15.37	9.91	-1.71	-0.81

2,2'-Dipyridyl amine						
20 mM modulator	6	0	3.70	0.53	0.43	-0.36
5 mM ALA + 20 mM modulator	64	30	20.19	20.67	-1.97	-1.01
30 mM modulator	9	0	0.94	0.09	0.60	-0.62
5 mM ALA + 30 mM modulator	82	15	15.48	10.80	-0.80	-0.92
Correlation coefficient	-	-	0.962	0.81	-	-
Level of significance	-	-	0.1%	5%	-	-
Solvent only	0	0	0.00	0.00	0.00	0.00
5 mM ALA	0	75	44.20	16.82	0.86	0.35
10 mM modulator	84	10	12.25	5.80	0.27	0.12
5 mM ALA + 10 mM modulator	87	80	36.11	17.70	0.78	0.24
phenanthridine						
20 mM modulator	92	15	8.98	6.67	0.01	-0.01
5 mM ALA + 20 mM modulator	94	70	49.60	29.79	0.31	-0.04
30 mM modulator	100	10	5.46	3.33	0.56	0.38
5 mM ALA + 30 mM modulator	100	80	57.05	72.84	1.87	1.76
Correlation coefficient	-	-	0.955	0.706	-	-
Level of significance	-	-	0.1%	10%	-	-

Table IV. Response of Cucumber, a DDV/LDV Plant Species and of Soybean, a DMV/LDV Plant Species to Inducers of DV Mg-protoporphyrin accumulation

Treatments, abbreviations and definitions are as in Table I. Adapted from (27).

Chlorophyll biosynthesis modulator	Treatment	Photodynamic damage (%)		Cucumber			
		Soybean	Cucumber	Pchlide		MPE	
				MV	DV	MV	DV
				Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)			
	Solvent only	0	0	0.00	0.00	0.00	0.00
	5 mM ALA	0	45	100.16	46.36	1.65	-0.58
	10 mM modulator	6	0	31.45	7.69	-0.86	-0.09
	5 mM ALA + 10 mM modulator	34	25	75.40	19.60	0.73	4.50
2,2'-dipyridyl	20 mM modulator	46	0	2.94	-4.76	1.39	15.29
	5 mM ALA + 20 mM modulator	53	90	28.94	37.20	5.34	70.39
	30 mM modulator	80	25	8.73	-3.96	6.07	15.32
	5 mM ALA + 30 mM modulator	90	100	45.16	29.73	14.30	160.04
	Correlation coefficient			0.329	0.7415	0.814	0.846
	Level of significance			n.s.	10%	5%	5%

to the untreated controls, and/or (b) when used in conjunction with
ALA, it should result in the inhibition of MV Pchlide accumulation
in comparison to the ALA-treated control.

4,7-phenanthroline, 2,3-diphyridyl and 2,4-dipyridyl (Table V)
as well as 1,7-phenanthroline (Table I C) fall into this group of
Chl biosynthesis modulators. In most cases so far investigated,
when the inhibitor was used jointly with ALA, especially at the
higher concentration levels of inhibitor, the inhibition of MV
Pchlide dark-accumulation was accompanied by an enhancement of DV
Pchlide accumulation, in comparison to the ALA-treated control
(Table V). Mg-protoporphyrin accumulation was not observed.

In cucumber, a DDV/LDV plant species, inhibitor-induced photo-
dynamic damage over and beyond the ALA-treated controls was either
minimal (4,7-phenanthroline, 2,3-diphyridyl in Table V and 1,7-
phenanthroline in Table I C) or was absent (2,4-dipyridyl in Table
V). However, in soybean a DMV/LDV plant species, these same ALA +
inhibitor treatments resulted in extensive photodynamic damage over
and beyond the ALA-treated controls (Table V). These results were
in turn fully compatible with the proposed mode of action
hypothesis.

Epilogue

The research effort described in this work has already led to the
development of photodynamic herbicide formulation capable of con-
trolling broad leaf weeds in Kentucky bluegrass, under field con-
ditions (47) and in controlling several monocot and dicot weed
species in corn and soybean under greenhouse conditions. In sum-
mary such an effort has involved (a) the classification of the
plant species to be destroyed and those to be saved into their
respective greening groups, (b) selection of one or more Chl bio-
synthesis modulators to act jointly with ALA and to induce the
undesirable weeds to accumulate undesirable tetrapyrroles that do
not belong to a functional biosynthetic route, (c) development of a
field solvent system capable of delivering the ALA and the Chl
biosynthesis modulator(s) to the chloroplast, where ALA is con-
verted to tetrapyrroles and finally (d) testing the developed
solvent system under the field conditions for which it had been
designed (47).

Because of the possibility of combining individual members of
the four classes of Chl biosynthesis modulators and ALA, five,
four, three or two at a time, it is possible to design a very large
number of useful herbicides. For example with the 13 Chl biosyn-
thesis modulators described in this work, it is already possible to
design 3458 different herbicidal mixtures. On the other hand the
discovery of one or two additional Chl biosynthesis modulators has
the potential of resulting in 1470 and 2410 additional herbicides
respectively.

Table V. Response of Cucumber a DDV/LDV Plant Species and of Soybean, a DMV/LDV Plant Species to Inhibitors of MV Protochlorophyllide Accumulation

Treatments, abbreviations and definitions are as in Table I. Adapted from (27).

Treatment	Photodynamic damage (%)		Cucumber			
			Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)			
			Pchlide		MPE	
	Soybean	Cucumber	MV	DV	MV	DV
Chlorophyll biosynthesis modulator						
Solvent only	0	0	0.00	0.00	0.00	0.00
5 mM ALA	3	60	41.65	8.04	0.92	0.91
10 mM modulator	93	3	-0.24	2.66	0.20	0.34
5 mM ALA + 10 mM modulator	94	45	19.18	16.00	1.05	0.19
20 mM modulator	100	21	-5.33	3.09	0.99	-0.95
4,7-phenan- throline						

5 mM ALA + 20 mM modulator	94	56	12.08	18.85	1.38	-0.45
30 mM modulator	100	48	0.74	14.60	0.86	0.19
5 mM ALA + 30 mM modulator	97	88	-4.58	25.70	1.21	-0.56
Correlation coefficient	-	-	0.299	0.889	-	-
Level of significance	-	-	n.s.	1%	-	-
2,3-dipyridyl						
Solvent only	0	0	0.00	0.00	0.00	0.00
5 mM ALA	8	49	65.42	30.80	-0.25	6.27
10 mM modulator	28	0	6.08	5.55	-0.22	1.48
5 mM ALA + 10 mM modulator	35	25	26.16	31.26	0.70	-0.31
20 mM modulator	40	0	-1.01	6.82	1.47	0.62
5 mM ALA + 20 mM modulator	45	28	41.56	58.53	-0.79	3.98
30 mM modulator	65	0	8.46	14.59	-0.14	1.47
5 mM ALA + 30 mM modulator	70	40	19.50	38.75	0.63	2.01
Correlation coefficient	-	-	0.869	0.763	-	-
Level of significance	-	-	5%	10%	-	-

Table V. Continued. Response of Cucumber, a DDV/LDV Plant Species, and of Soybean, a DMV/LDV Plant Species, to Inhibitors of MV Protochlorophyllide Accumulation

Chlorophyll biosynthesis modulator	Treatment	Photodynamic damage (%)		Cucumber			
				Exogenous ALA-induced tetrapyrrole accumulation (nmoles per 100 mg protein)			
				Pchlide		MPE	
		Soybean	Cucumber	MV	DV	MV	DV
	Solvent only	3	0	0.00	0.00	0.00	0.00
	5 mM ALA	8	35	19.65	8.15	0.93	-1.12
	10 mM modulator	13	0	-3.32	-0.63	0.32	-1.03
	5 mM ALA + 10 mM modulator	25	-	-	-	-	-
	20 mM modulator	20	0	-5.05	-0.12	1.32	-0.46
	5 mM ALA + 20 mM modulator	40	8	9.28	11.63	-0.16	0.57
	30 mM modulator	47	0	-5.71	4.15	0.57	0.82
2,4-dipyridyl	5 mM ALA + 30 mM modulator	60	5	3.44	26.54	0.34	0.30
	Correlation coefficient			0.927	0.227	-	-
	Level of significance			5%	n.s.	-	-

Legend of Symbols. Pchlide: protochlorophyllide; Proto: protopor-
phyrin IX; Mg-proto; Mg protoporphyrin IX; MPE: Mg-protoporphyrin
monoester; MP (E): a mixture of Mg-Proto and MPE; Chl: chloro-
phyll; monocot: monocotyledonous plant; dicot: dicotyledonous
plant; MV: monovinyl; DV: divinyl; Chlide: chlorophyllide; Proto-
gen: protoporphyrinogen; Alk. E: alkyl ester; Pchl: protochloro-
phyllide ester; Pchl(ide): pchlide + Pchl; DPy: 2,2'-dipyridyl.

Literature Cited

1. Rebeiz, C. A.; Montazer-Zouhoor, A.; Hopen, H. J.; Wu, S. M.
    Enzyme Microb. Tecnol. 1984, 6, 390-401.
2. Hopf, F. R.; Whitten, D. G. In The Porphyrins; Dolphin, D.,
    Ed.; Academic: New York, 1978; Vol. 2, pp 161-195.
3. Foote, C. S. In Porphyrin Localization and Treatment of
    Tumors; Alan R. Liss: New York, 1984; pp 3-18.
4. Mattheis, J. R.; Rebeiz, C. A. J. Biol. Chem. 1977, 252,
    4022-4024.
5. Mattheis, J. R.; Rebeiz, C. A. J. Biol. Chem. 1977, 252,
    8347-8349.
6. Mattheis, J. R.; Rebeiz, C. A. Photochem. Photobiol. 1978,
    28, 55-60.
7. Hougen, C. L.; Meller E.; Gassman, M. L. Plant Science
    Letters 1982, 24, 289-294.
8. Rebeiz, C. A.; Mattheis, J. R.; Smith, B. B.; Rebeiz C. C.;
    Dayton, D. F. Arch. Biochem. Biophys. 1975, 171, 549-567.
9. Smith, B. B.; Rebeiz, C. A. Photochem. Photobiol. 1977, 26,
    527-532.
10. Bazzaz, M. B.; Rebeiz, C. A. Photochem. Photobiol. 1979, 30,
    709-721.
11. Rebeiz, C. A.; Daniell, H.; Mattheis, J. R. In 4th Symposium
    on Biotechnology in Energy Production and Conservation; Scot,
    C. D., Ed.; John Wiley: New York, 1982; pp 413-439.
12. Sisler, E. C.; Klein, W. Physiol. Plant. 1963, 16, 315-322.
13. Rebeiz, C. A.; Abou Haidar, M.; Yaghi, M.; Castelfranco, P. A.
    Plant Physiol. 1970, 46, 543-549.
14. Rebeiz, C. A.; Mattheis, J. R.; Smith, B. B.; Rebeiz, C. C.;
    Dayton, D. F. Arch. Biochem. Biophys. 1975, 166, 446-465.
15. Granick, S.; Mauzerall, D. In Metabolic Pathways;
    Greenberg, D. M., Ed.; Academic: New York, 1961; pp 525-615.
16. Rebeiz, C. A.; Castelfranco, P. A. Plant Physiol. 1973, 24,
    129-172.
17. Lascelles, J. In Porphyrins and Related Compounds;
    Goodwin, T. W., Ed.; Academic: New York, 1968; pp 49-59.
18. Rebeiz, C. A.; Lascelles, J. In Photosynthesis: Energy
    Conversion by Plants and Bacteria; Govindjee, Ed.; Academic:
    New York, 1982; Vol. 1, pp 699-780.
19. Rebeiz, C. A.; Yaghi, M.; Abou-Haidar, M.; Castelfranco, P. A.
    Plant Physiol. 1970, 46, 57-63.
20. Daniell, H.; Rebeiz, C. A. Biochem. Biophys. Res. Commun.
    1982, 104, 837-843.
21. Daniell, H.; Rebeiz, C. A. Biochem. Biophys. Res. Commun.
    1982, 106, 466-470.
22. Daniell, H.; Rebeiz, C. A. Biotech. Bioeng. 1984, XXII,
    481-487.
23. Granick; S. J. Biol. Chem. 1950, 183, 713-730.

24. Castelfranco, P. A.; Beale, S. I. In The Biochemistry of
     Plants; Hatch, M. D.; Boardman, N. K., Eds.; Academic: New
     York, 1981; Vol. 8, pp 375-421.
25. Rebeiz, C. A.; Belanger, F. C.; McCarthy, C. A.;
     Freyssinet, G.; Duggan, J. X.; Wu, S. M.; Mattheis, J. R. In
     Photosynthesis. Chloroplast Development; Akoyunoglou, G.,
     Ed.; Balaban International Science Services: Philadelphia,
     1981; Vol. 5, pp 197-212.
26. Rebeiz, C. A.; Wu, S. M.; Kuhadja, M.; Daniell, H.;
     Perkins, E. J. Mol. Cell. Biochem. 1983, 57, 97-125.
27. Rebeiz, C. A.; Montazer-Zouhoor, A.; Mayasich, J. M.;
     Tripathy, B. C.; Wu, S. M.; Rebeiz, C. C. Crit. Rev. Plant
     Lei. In press.
28. McCarthy, S. A.; Belanger, F. C.; Rebeiz, C. A. Biochemistry
     1981, 20, 5080-5087.
29. Belanger, F. C.; Rebeiz, C. A. J. Biol. Chem. 1982, 257,
     1360-1371.
30. Belanger, F. C.; Rebeiz, C. A. Biochemistry 1980, 19,
     4875-4883.
31. Belanger, F. C.; Rebeiz, C. A. Plant Sci. Lett. 1980, 18,
     343-350.
32. McCarthy, S. A.; Mattheis, J. R.; Rebeiz, C. A. Biochemistry
     1982, 21, 242-247.
33. Belanger, F. C.; Rebeiz, C. A. J. Biol. Chem. 1980, 255,
     1266-1272.
34. Duggan, J. X.; Rebeiz, C. A. Plant Sci. Lett. 1982, 24,
     27-37.
35. Wu, S. M.; Rebeiz, C. A. Tetrahedron 1984, 40, 659-664.
36. Belanger, F. C.; Duggan, J. X.; Rebeiz, C. A. J. Biol. Chem.
     1982, 257, 4849-4858.
37. Duggan, J. X.; Rebeiz, C. A. Plant Sci. Lett. 1982, 27,
     137-145.
38. Rebeiz, C. A.; Tripathy, B. C.; Wu, S. M.; Montazer-
     Zouhoor, A.; Carey, E. E. In Regulation of Chloroplast
     Differentiation; Akoyunoglou, G.; Senger, H., Eds.; Alan R.
     Liss: New York, 1986; pp 13-24.
39. Tripathy, B. C.; Rebeiz, C. A. J. Biol. Chem. 1986, 26
     13556-13564.
40. Tripathy, B. C.; Rebeiz, C. A. Anal. Biochem. 1985, 14,
     43-61.
41. Carey, E. E.; Tripathy, B. C.; Rebeiz, C. A. Plant Physiol
     1985, 79, 1059-1063.
42. Carey, E. E.; Rebeiz, C. A. Plant Physiol. 1985, 79, 1-6.
43. Rebeiz, C. A.; Montazer-Zouhoor, A.; Rebeiz, C. C. In Thirty
     Eighth Illinois Custom Spray Operators Training Manual,
     University of Illinois Cooperative Extension Service, Ed.;
     Univ. Illinois Press: Urbana, IL, 1986; pp 91-93.
44. Jones, O. T. G. Biochem. J. 1963, 88, 335-343.
45. Duggan, J.; Gassman, M. Plant Physiol. 1974, 53, 206-215.
46. Bednarick, D. P.; Hoober, J. K. Arch. Biochem. Biophys. 1985,
     240, 369-379.
47. Rebeiz, C. A.; Rebeiz, C. C.; Montazer-Zouhoor, A. American
     Lawn Applicator. 1987, In Press.

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