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ABSTRACT

The mechanism of photosensitization by polyacetylenes and biosynthetically derived thiophenes from species of the plant family Asteraceae was examined. With thiophenes photosensitization of S. cerevisiae and E. coli occurred under aerobic but not anaerobic conditions. In similar experiments with polyacetylenes, both photodynamic and non-photodynamic mechanisms were observed. While the relative toxicites of thiophenes and polyacetylenes under near UV radiation were generally similar, the in vitro generation of singlet oxygen was considerably less for polyacetylenes than thiophenes, which is additional evidence for the existence of an alternative mechanism of action in polyacetylene photosensitization. In vitro rates of photodegradation of polyacetylenes were found to be higher than for thiophenes suggesting that bond breaking/formation processes are more favored relative to energy transfer to oxygen for polyacetylenes than for thiophenes. An assessment of the relative phototoxicities of the test substances revealed a positive correlation between phototoxicity and partition coefficient with yeast but not E. coli. In addition thiophenes were found to be more phototoxic and to have higher partition coefficients than acetylenes. Thiophenes were also found to have a higher relative light absorption than acetylenes. Otherwise there was little correlation between phototoxicity and photon absorption. Additional structure function relationships are discussed.

Résumé

Nous avons étudié le mécanisme de la photosensibilisation par les polyacétylènes et les thiophènes dérivés biosynthétiquement d'espèces végétales de la famille des Astéraceae. En présence de thiophènes, la photosensibilisation de S. cerevisiae et d'E. coli s'est produite dans des conditions aérobiques mais non dans des conditions anaérobiques. Lors d'expériences similaires en présence de polyacétylènes, nous avons observé à la fois les mécanismes photodynamique et non photodynamique. Alors que les toxicités relatives des thiophènes et des polyacétylènes exposés à des radiations du proche UV sont généralement semblables, la production in vitro d'oxygène singlet par les polyacétylènes a été considérablement moindre que celle générée par les thiophènes: c'est là une preuve supplémentaire de l'existence d'un autre méchanisme d'action dans la photosensibilisation par les polyacétylènes. In vitro, les taux de photodégradation des polyacétylènes se sont montrés plus élevés que ceux des thiophènes, suggérant que les processus de bris/formation de liaisons, plutôt que le transfert d'énergie à l'oxygène sont davantage favorisés dans le cas des polyacétylènes que dans celui des thiophènes. Une étude des phototoxicités relatives des substances testées a démontré une corrélation positive entre la phototoxicité et le coefficient de partition chez la levure mais non chez E. coli. De plus, nous avons noté que les thiophènes sont plus phototoxiques et ont des coefficients de partition plus élevés que les acétylènes. Nous avons également observé que l'absorption relative de la lumière par les thiophènes est supérieure à celle des acétylenes. D'autre part il n'y a eu que peu de corrélation entre la phototoxicité et l'absorption photonique. D'autres relations structure/fonction sont également discutées.

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List of Abbreviations

A	absorbance
A^{+}, A^{-}	substrate free radicals
A_{ox}	fully oxidized substrate
A_s	substrate
BLB	blacklight-blue (light source)
C_{10} (#)	carbon skeleton chain length
DABCO	1,4-diazabicyclo [2,2,2] octane
DMFu	2,5-dimethylfuran
D_2O	deuterated water
ESR	electron spin resonance
EtOH	ethanol, 95% unless otherwise specified
HPLC	high performance liquid chromatography
$h\nu$	light (Planck's constant x propagation velocity of light)
I_a	absorbed intensity
I_o	incident intensity
k'	first order rate constant for $^1O_2$ production
K^+	potassium ion
Log K'	log of the capacity factor (K')
Log P	log of the water/octanol partition coefficient (P)
Log P_{cal}	calculated log of the partition coefficient from log K'
M	moles
MCD	magnetic circular dichroism (spectroscopy)
MeOH	methanol, 100% unless otherwise specified
MOA	mechanism of action
N_2	molecular nitrogen
N_3	azide (sodium, unless specified)
NMR	nuclear magnetic resonance (spectroscopy)
O.D.	optical density
ODS	octadecylsilane (HPLC column packing)
·OH	hydroxyl free radical
O_2	molecular oxygen

$^1O_2$	singlet (excited) state molecular oxygen
$^3O_2$	triplet (ground) state molecular oxygen
$O_2^{\overline{\cdot}}$	superoxide free radical
P.E.	petroleum ether
PHD-ol	phenylheptadiyne-alcohol (compound VIII)
PHT	phenylheptatriyne (compound V)
r	correlation coefficient
r^2	correlation coefficient squared
rpm	revolutions per minute
RP-C_{18}	HPLC reverse-phase column
$^1S^*$	singlet excited state of a sensitizer
$^3S^*$	triplet excited state of a sensitizer
S_o	ground state of a sensitizer
SOD	superoxide dismutase
SP	secondary product(s)
SPS	secondary plant substance(s)
t_R	retention time of a compound by HPLC
t_o	retention time of a non-absorbed carrier solvent
T_1	lowest excited triplet state
UV	ultraviolet
V_o	initial rate of photodegradation
α-T	alpha-terthienyl (compound III)
$^1\Delta_g$	singlet excited state, spins paired
$^1\Sigma_g$	triplet excited state, spins unpaired
ϵ	extinction coefficient
λ	wavelength
λ_{max}	wavelength of maximum absorbance
μ	microns
π	antisymmetrical orbital about the bond axis
ϕ	quantum yield for singlet oxygen

INTRODUCTION

i) Plant secondary products as protective agents with reference to acetylenes:

Plants have adopted a chemical defense strategy for protection against the attack from potential pathogens, herbivores and competitors. The defense compounds elaborated by the plant belong to diverse chemical groups which were originally designated "secondary metabolites" so named since they seemed to lack an obvious primary metabolic function and appeared to be waste products sequestered in the plant. Today these compounds are considered to be " functional secondary products",(Swain, 1977), dynamic within the plant and in flux with environmental conditions.

In higher plants and fungi there are over ten thousand known secondary products which are divided into a number of classes (Table 1) based on their chemical structure. Any one plant may contain a large number of those compounds from several chemical classes, all of which together enhance the survival of the plant. Many plant substances can perform a number of ecological functions within the plant, affording protection to the plant from a number of detrimental sources. In such cases the real selective advantage for particular secondary substances may be difficult to discern, especially since present day selective pressures may not be representative of the evolutionary selective pressures which

Table 1 Chemical classes of secondary plant substances.

Chemical Class	No. of known structures	Examples	Protection against:*
Acetylenes	750	Wyerone	Fungi
Alkaloids	4500	Lupanine	Mammals
Amino acids	250	Canavanine	Insects
Carotenoids	300	β-Carotene	Photoprotection
Coumarins	150	Scopoletin	Fungi
Cyanogenic glycosides	50	Linamarin	Molluscs
Flavonoids	1200	Morin	Insects
Glucosinolates	80	Sinigrin	Insects
Lignans	50	Excelsin	Insects
Lipids	100	Waxes	Fungi
Phenolic acids	100	Vanillic acid	Plants
Polyketids	500	Hircinol	Fungi
Quinones	200	Juglone	Plants
Terpenes	1100	Glaucolide-A	Insects
Steroids	600	Ecdysones	Insects
Miscellaneous	500	Tuliposide	Fungi
Proteins	?	Lectins	Insects
Polysaccharides	?	Acylated polysaccharides	Fungi
Other polymers	?	Cutin	Fungi

* Only one example given, others are possible.

(Table adapted from Swain, 1977.)

established the metabolic machinery to elaborate particular classes of defense and communication substances in various plant lineages (Janzen, 1979). It is this early establishment and maintenance of particular metabolic machinery in specific plant lineages that has led to the strong phylogenetic affinities of plant secondary products. This has made secondary substances important to plant taxonomists who have been largely responsible for the identification of these substances in many chemical classes. Thus in many cases the ecological significance of a large number of plant secondary substances may not have been examined, and this field has only become an important research area in recent years. For example polyacetylenes which form the basis of this present study are a large group of 750 secondary substances whose chemistry and taxonomy has been examined in over 500 published papers since 1900 (Bohlmann *et al*., 1973). Less than 10% of these articles have explored the physiological effects of these compounds and the discovery that their biological activity is enhanced many times in the presence of light was not published until 1975 by Camm *et al*. It is this phototoxic activity that is examined in the investigation presented here.

ii) Phytoalexins and disease resistance substances:

Plants are in constant contact with a large variety of microorganisms and it is therefore surprising that generally plants do not succumb to microbial infections. This is in part due to the presence of fungistatic and bacteriostatic compounds on the surface of the plant and the inability of most microorganisms to penetrate the cutin or suberin layers. In the event of subcuticular invasion there may be further, more toxic, compounds present or the invasion and subsequent necrosis of cells may induce *de novo* synthesis of antibiotic defence compounds (Swain, 1977).

Ingham (1973) divides disease resistance substances in plants into four catagories: 1) Prohibitins, which are pre-infectional metabolites present in the plant which reduce or completely halt the *in vivo* development of microorganisms; 2) Inhibitins, are compounds which are normally present in the plant but undergo a post-infectional increase in order to express fully their toxic potential; 3) Post-inhibitins, which are post-infectional metabolites formed by the hydrolysis or oxidation of pre-existing substances resulting in increased toxicity; 4) Phytoalexins, which are compounds that are enzymatically synthesised *de novo* or are normally found in extremely low concentrations. The phytoalexins are strictly post-infectional metabolites whose formation involves gene

derepression or the activation of a latent enzyme system.

In any one plant all of these systems of defense may be potentially operative. It is not possible energetically or with regards to the risk of autotoxicity for plants to produce sufficient quantities of toxic compounds to provide complete protection of all tissues. Instead plant secondary compounds occur in varying amounts in different parts of the plant at different times as a "chemical defense investment". The apogee then of a minimal chemical defense investment must be the phytoalexin.

As stated earlier, examples of phytoalexins can be found in almost all classes of secondary plant substances although the largest demonstrated number have come from the pterocarpins and isoflavans (reviewed by Van Etten, 1976). More recently sesquiterpene compounds from the Solanaceae (Stoessl et al., 1976) and acetylenic compounds such as wyerone (broad bean, Vicia faba; Hargraves et al., 1976); safynol & dehyrosafynol (safflower, Carthamus tinictorius; Allen & Thomas, 1971 a & b); falcarindiol, falcarinol and cis-tetradeca-6-ene-1,3-diyne-5,8-diol (tomato leaves, Lycopersicon esculentum; DeWit & Koddle, 1981), have been discovered (structures in Figure 1). Reisch et al., (1967) investigated the bacteriostatic and fungistatic effects of a large number of simple synthetic acetylenes and concluded that fungistatic effects increased with polarization of the triple bond and with increased lipid solubility. Alternatively bacteriostatic

Figure 1. Chemical structures for some known acetylenic phytoalexins. a) wyerone, (Fawcett et al., 1968); b) dehydrosafynol, & c) safynol, (Allen & Thomas, 1971a & b); and d) falcarindiol, e) falcarinol, & f) cis-tetradeca-6-ene-1,3-diyne-5,8-diol, (de Wit & Kodde, 1981).

a) $CH_3-CH_2CH=CH-C\equiv C-C(=O)-C_4H_2O-CH=CH-COOH$ (2,5-disubstituted furan ring)

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

c) $CH_3-CH=CH-(C\equiv C)_3-CH=CH-CH(OH)-CH_2(OH)$

d) $CH_3-(CH_2)_5-CH_2-CH=CH-CH(OH)-(C\equiv C)_2-CH(OH)-CH=CH_2$

e) $CH_3-(CH_2)_5-CH_2-CH=CH-CH_2-(C\equiv C)_2-CH(OH)-CH=CH_2$

f) $CH_3-(CH_2)_5-CH(OH)-CH=CH-CH(OH)-(C\equiv C)_2-H$

effects were enhanced with water solubility. They were not aware of the phototoxicity of acetylenes though, which could alter their findings and makes reassessment of all work on polyacetylene phytoalexins necessary (Towers & Wat, 1978).

More recently other secondary plant compounds have been shown to have increased fungal toxicity in the presence of light. A number of polyacetylenes have been shown to be phototoxic to microorganisms, (Camm et al., 1975) including several species of fungi (Towers, 1981). The furoquinoline alkaloid, dictamnine, formed photoadducts in fungal DNA (Pfyffer & Towers, 1982) and the beta-carboline alkaloids were found to be phototoxic to yeast (McKenna et al., 1981), although their role as phytoalexins is yet to be determined. Recently Bakker & coworkers (1983) reported on the photoactivity of several isoflavonoids. They found that five of six commonly known isoflavonoid phytoalexins photoinactivated glucose-6-phosphate dehydrogenase in an in vitro assay and implicated "sensitizer free-radicals" as a mechanism of action from ESR (electron spin resonance) spectroscopy. The presence of these natural sensitizing compounds from plants is suggestive that this may be a major advance in the chemical warfare between plants and pathogens which until recently had not been examined.

iii) Allelopathy:

The idea that some plants excrete substances from their roots injurious to other plants was first suggested by De Candolle in 1832, although the term "allelopathy" was not coined until 1937 by Molisch. Generally today the term allelopathy refers to the detrimental effects of one plant species on another plant species, often involving a gross reduction in the germination efficiency, growth and development of the recipient plant (Wittaker, 1970). These detrimental effects have been shown many times to be mediated by the release of phytotoxic secondary substances by the donor plant (Rice, 1964). Various modes of release of these substances have been documented including volatilization, surface washing, active exudation from roots & leaves or leaching from its litter. Often further modification by environmental factors and/or microbial activity is necessary to confer toxicity to the substance.

In some cases allelopathic substances may be toxic to the donor plant and play significant roles in plant succession (Rice, 1964) where a pioneering plant species suffers from autotoxicity after several generations allowing other plant species to become established. This has been demonstrated for a number of Asteraceae pioneering species. The possible allelopathic effects of the polyacetylene phenylheptatriyne and the acetylenic derivative alpha-terthienyl were examined by Campbell _et al_., 1982.

This work and the work of Kobayashi & coworkers (1980) on allelopathic C_{10} acetylenes from Asteraceae species is suggestive that some polyacetylenic compounds may be effective allelopathic agents.

iv) Insect deterrents:

In nature hardly any plant species can be found without some insect species feeding on it, which indicates that in each particular case the insect has found a way to circumvent deterrent effects of particular secondary plant substances (Schoonhoven, 1972). It is highly likely that without such compounds the majority of higher plant species could not withstand the destructive appetites of the many species of herbivorous insect pests. Undoubtedly this herbivory has been a strong selection factor for plant secondary compounds and new toxic strategies. Recent work has suggested that certain secondary metabolites from plants are capable of photosensitizing insects, which appears to be of ecological, evolutionary and physiological significance in the chemical warfare between plants and insects (Arnason et al., 1983). Such compounds include the furanocoumarins (Berenbaum, 1978 & 1981), furanoquinoline alkaloids (Arnason & Towers, unpublished results), beta-carboline alkaloids (Arnason & Towers, unpublished results), the isoquinoline alkaloid, berberine (Philogene

et al., 1984), polyacetylenes & their thiophene derivatives (Arnason et al., 1981b & Champagne et al., 1983) and extended quinones. In addition to direct lethal effects to insects some of these photosensitizing compounds can act as feeding deterrents and retard larval development as found in the case of the thiophene alpha-terthienyl (Champagne et al., 1983) and the acetylene phenylheptatriyne (McLachlan et al., 1982). Photosensitizers may also be ovicidal and play a major role in determining oviposition in insects (Kawazu et al., 1977), although this has not been extensively examined. Some compounds such as -T found in the roots of marigolds (*Tagetes* sp.) are extremely phototoxic to soil nematodes (Gommers et al., 1980; Gommers, 1972).

Of course some specialist insects have adapted to the presence of natural photosensitizers, such as the leaf rolling habit of some microlepidoptera on phototoxic Apiaceae species avoiding sensitizing near-UV light (Berenbaum, 1978). Other insect species can actually modify or detoxify the photosensitizer such as the larvae of the black swallowtail butterfly, *Papilio polyxenes* which detoxifies linear furanocoumarins of the Apiaceae, (Ivie et al., 1983), but cannot detoxify angular furanocoumarins (Berenbaum & Feeny, 1981) from the same family. The angular furanocoumarins are found only in the most advanced tribes of the Apiaceae and are examples of evolutionary response to the selective pressures of insect

herbivory.

The role of naturally occurring polyacetylenes as phototoxic agents against insects has only recently been examined for a few selected compounds. Further work in this field may demonstrate that there is a wide spread ecological significance for polyacetylenes as deterrents against insect herbivory.

v) Distribution of acetylenes in the plant kingdom:

Acetylene compounds are distributed throughout the plant kingdom from the simplest microorganisms to the most advanced plant family, Asteraceae. It should be noted that this is not a continuous distribution, nor that the Asteraceae are closely linked to microorganisms or algae, but rather that the ability to form acetylenic compounds has arisen several times within the plant kingdom. Each group in fact has its own characteristic acetylenes, which exemplifies the polyphyletic nature of acetylene formation in different plant groups.

Considering the lower plant groups, acetylenic compounds have been found in fungi and algae. There are approximately sixty polyacetylenes known from the true fungi, nearly all of which occur in the class Basidiomycetes. These acetylenes are mainly of C_{10} origin and similar to those of higher plants, although

generally they are of the trans configuration and highly polar (Bohlmann et al., 1973). In the algae the families: Chrysophyceae, Bacillariophyceae and Xanthophyceae produce acetylenic carotenoids containing one or two acetylenic bonds. The family, Rhodomelaceae (red algae) though produce unusual monoacetylenes based on an eight membered ring structure (Bohlmann et al., 1973).

In the higher plants there are approximately seven hundred or more acetylenic compounds known. These compounds are generally C_{10}, C_{13}, C_{17}, or C_{18} structures, with up to five acetylenic bonds present. Many compounds contain ring structures such as phenyl-rings, thiophenes, furans, and spiroketalenoethers. In addition several end groups are possible including methyl (CH_3), alcohol (CH_2OH), aldehydes (CHO), esters ($COOCH_3$) and acetates (CH_2OCOCH_3). In some instances the compounds can be halogenated. Some of these variations have been examined in this study and are shown in Figure 4. These are distributed throughout 20 higher plant families, although polyacetylenes are regularly found only in the families: Araliaceae, Campanulaceae, Santalaceae, Umbelliferae and Asteraceae, with by far the largest number from the Asteraceae. The polyacetylenes from the Asteraceae are unique in that there are many aromatic and heterocyclic compounds, which appear to be more advanced structures. These compounds have not been reported to have hormonal activity in plants nor are they essential primary

metabolites, (Towers & Wat, 1978). As described earlier they do appear to be very effective in protecting the plant from various predators, pathogens & competitors.

vi) Distribution of polyacetylenes in the plant:

Polyacetylenes have been found in all parts of the plant (Bohlmann et al., 1973), but within the plant particular compounds may be restricted to specific organs or parts of organs (Camm et al., 1975; Towers & Wat, 1978). The same compounds may not be present in the same organs in different species, ie. phenylheptatriyne (PHT) is located in the leaves of Bidens pilosa but absent from the roots, while in some Coreopsis species this compound is the major compound in the roots (Towers & Wat, 1978). The actual site of synthesis for these compounds is unknown at present, although resin canals are often found in species containing polyacetylenes which may be the sites of synthesis (Towers & Wat, 1978). Recently an acetate esterase was isolated from Tagetes patula which is involved in the transformation of an acetylenic compound (Sutfeld & Towers, 1982). The enzyme had a high substrate specificity to 5-(4-acetoxy-1-butinyl) -2,2'-bithiophene, a compound which polymerizes spontaneously to give insoluble products. The activity of the enzyme produces 5-(4-hydroxy-1-butinyl)-2,2'-bithiophene which is stable.

This enzyme may be important in the synthetic pathway towards more stable thiophenes (Sutfeld & Towers, 1982), which the plant can sequester for chemical defense purposes. Other such enzymes are undoubtedly present within polyacetylene containing plants which aid in the synthesis of more stable compounds from less stable intermediates. The specificity of certain enzymes to particular organs could account for the specificity of some compounds to particular organs.

vii) Photosensitization:

An all encompassing definition of photosensitization is: the action of a component (photosensitizer) of a system that causes another component of the system to react to light (Turro & Lamola, 1977). The study of photosensitization is generally considered to have been started by Raab, (1900), who showed that acridine dyes in the presence of visible light killed paramecium (Spikes, 1977). Since the work of Raab there have been over 400 compounds found which photosensitize biological systems and more are being discovered every year (Spikes, 1977). Some common dye sensitizers include: methylene blue, toluidine blue, rose bengal, eosin Y, and acridine orange. Some naturally occurring biological photosensitizers include: hematoporphyrin, hypericin, chlorophyll, riboflavin and

psoralen. The structures for these compounds are shown in Figure 2.

The basis of photosensitization is the absorption of a quantum of light by a molecule which causes it to become electronically excited, moving electrons into higher orbitals. Molecules in excited electronic states have quite different chemical and physical properties than in their ground state and they are much more reactive, generally giving different products than thermal reactions involving only ground state molecules. It is the relaxation of the excited state (the shedding of its excess electronic energy) through several possible, often competing, pathways which is responsible for photosensitization reactions.

The loss of excitation energy can be divided into three broad catagories: 1) radiative relaxation, such as fluorescence & phosphorescence, where photons of light are emitted; 2) radiationless relaxation, such as internal conversion & intersystem crossing, where vibrational energy is absorbed by the molecule; and 3) photochemical processes. Radiative relaxation processes are generally slow processes in relation to other relaxation processes, phosphorescence being slower than fluorescence. Internal conversion occurs when vibrational energy is absorbed within the molecular matrix lowering the electronic state of the molecule but retaining its spin multiplicity. Intersystem crossing is the analogous process but takes place between excited electronic states of different multiplicities ($S_1 \longrightarrow T_1$ &

FIGURE 2. Chemical structures for some common dye (a-e) and naturally occurring (g-i) photosensitizers. a) methylene blue; b) toluidine blue; c) rose bengal; d) eosin Y; e) acridine orange; f) chlorophyll a; g) hypericin; h) riboflavin; & i) psoralen.

a) $(CH_3)_2N$ S N $N(CH_3)_2$

b) H_3C H_2N N S $N(CH_3)_2$

c) NaO I I O I O I Cl COO^- Cl Cl Cl

d) NaO Br Br O Br O Br COO^-

e) CH_3 N CH_3 N N CH_3 CH_3

f) OH O OH H_3C OH H_3C OH OH O OH

g) CH_2 CH CH_3 H_3C CH CH N N Mg N N H_3C CH_3 CH_2 R CH_2 O C=O O phytol R= COOCH

h) CH_2OH $(CHOH)_3$ CH_2 Me N N O NH Me N O

i) O O O

T⟶So). These two non-radiative relaxation processes occur very rapidly and can compete with photochemical processes.

In addition to photophysical relaxation processes electronically excited molecules can react with other molecules to obtain various stable end products which may return the excited molecule to its ground state or result in its consumption. The simplest non-radiative process which conserves the sensitizing molecule is the one-step radiationless energy transfer between an excited sensitizer and an acceptor molecule. Such a system is a resonance process and therefore is under normal selection rules and occurs largely from the triplet state (T_1) of the sensitizer.

It is this energy transfer process which results in the production of singlet oxygen ($^1O_2$), a major photosensitizing intermediate in biological systems. This process is facilitated by the fact that ground state oxygen is a triplet state and reqires only 23 kcal to reach the long lived first singlet excited state (with paired electrons) designated $^1\Delta_g$. The second singlet excited state $^1\Sigma_g$ requires 37 kcal but electron spins are not paired and it readily converts to $^1\Delta_g$ via internal conversion processes. This energy transfer process with $^3O_2$ can be written as:

$$S_o \longrightarrow {}^1S^* \longrightarrow {}^3S^* \qquad (1)$$

$$^3S^* + {}^3O_2 \longrightarrow S_o + {}^1O_2 \qquad (2)$$

$$^{1}O_2 + A_s \longrightarrow Aox \qquad (3)$$

where singlet oxygen results in an oxidized substrate (Aox) in a biological system. This process is commonly referred to as a Type II photodynamic reaction and is the major photosensitization process in biological systems.

Alternatively an excited sensitizer could undergo an electron transfer process (type I reaction) with another component (A) where the primary process is:

$$S^* + A_s \longrightarrow S^{\dot{+}} + A^{\dot{-}} \qquad (4)$$

or

$$S^* + A_s \longrightarrow S^{\dot{-}} + A^{\dot{+}} \qquad (5)$$

In this photochemical process there is often the formation of a complex called an exciplex, which occurs between the excited sensitizer and the substrate. The ground state sensitizer is regenerated from S^- by reacting with oxygen to give a superoxide radical $O_2^{\dot{-}}$. The O_2 produced thus, can react directly with the substrate to fully oxidize it:

$$S^{\dot{-}} + {}^{3}O_2 \longrightarrow {}^{3}S + O_2^{\dot{-}} \qquad (6)$$

$$O_2^{\dot{-}} + A_s \longrightarrow Aox \qquad (7)$$

Alternatively:

$$S^{\dot{+}} + A_s \longrightarrow S_0 + A^{\dot{+}} \qquad (8)$$

$$A^{\ddagger} + {}^3O_2 \longrightarrow A_{ox} \qquad (9)$$

In these free radical mechanisms the sensitizer-substrate specificity is critical but in view of the reactivity of membrane components comprised of proteins, sterols and unsaturated fatty acids, both singlet oxygen and free radical mechanisms are anticipated to play a crucial role in photosensitizations (Ito, 1983).

Other types of photochemical reactions not involving oxygen are possible such as linear and cycloadditons to unsaturated systems, substitution reactions, cis-trans isomerizations, oxygen-independent free-radical generation, structural rearrangements, fragmentation, or polymerization (Turro & Lamola, 1977). In secondary plant substances photosentizations not involving oxygen, the cycloaddition of furanocoumarins (Musajo et al., 1974) and the furoquinoline alkaloid, dictamnine (Pfyffer & Towers, 1982; Pfyffer et al., 1982) to DNA have been well documented. In this mechanism the compounds dark intercalate into the DNA helix such that one or two of the double bonds of the ring structure align with pyrimidine double bonds of the DNA helix. The double bonds undergo cycloaddition via the excited triplet state to the pryimidine substrate molecule resulting in the formation of a photoadduct (Figure 3). Compounds such as dictamnine, sterically hindered linear furanocoumarins and angular furanocoumarins are capable of forming only monofunctional adducts, while other

FIGURE 3. A schematic diagram of photoinduced furanocoumarin cross-linking of the DNA helix at thymine residues. The absorption of an intial photon of light may form either a 3,4 or a 4',5' monocycloadduct. The absorption of a second photon, hν, after the formation of the 4',5' monofunctional adduct may produce a 3,4-4',5' dicycloadduct crosslinking the DNA helix. This can occur only if thymine residues are opposite on adjacent ladder rungs of the helix (see inset diagram) and only for linear furanocoumarins which are not sterically hindered. Sterically hindered linear furanocoumarins and angular furanocoumarins can only produce monofunctional adducts. The inset diagram shows the positioning of the furanocoumarin molecule (the heavy horizontal lines) and possible cycloaddition bridges (heavy vertical lines) within the DNA helix. (P represents the phosphate group and dr is the deoxyribulose sugar molecule; C, G, A, & T represent the nucleic acids cytosine, guanine, adenine & thymine respectively.) (Scheme after Musajo et al., 1974).

Psoralen + Thymine

hν

3,4 monocycloadduct

4',5' monocycloadduct

+ hν

3,4, 4',5' dicycloadduct

P >dr - C — G - dr< P
P >dr - A — T - dr< P
P >dr - T — A - dr< P
P >dr - C — G - dr< P
P >dr - G — C - dr< P
P >dr - A — T - dr< P
P >dr - A — T - dr< P
P >dr - C — G - dr< P

linear furanocoumarins, such as psoralen & 8-methoxypsoralen, form difunctional adducts or DNA crosslinks between the strands of DNA.

Recently it has been shown that several isoflavonoid phytoalexins photosensitize the enzyme glucose-6-phosphate dehydrogenase by an oxygen-independent free-radical mechanism (Bakker et al., 1983). This is suggestive that other secondary plant substances may also produce free-radicals under appropriate conditions which could enhance their toxicity and hence their protective role in the plant.

Although the other oxygen-independent reactions listed, including linear additions to unsaturated systems, substitution reactions, structural rearrangements & fragmentation could occur in biological systems, they have not been reported as photosensitization mechanisms for secondary plant products.

viii) Elucidating the photosensitization MOA:

The elucidation of the mechanism of photosensitization can be broken down into five steps: 1) Determine if the photosensitizer absorbs the exciting light, by comparing the action spectrum to the absorption spectrum of the compound; 2) Determine whether or not the photosensitizer is consumed in the reaction; 3) Determine if an electronic

energy transfer is involved or if 4) an electron transfer is involved in the photosensitization; 5) Determine if molecular oxygen is involved in the system (Turro & Lamola, 1977). The last step, the verification of the role of O_2, is important because most photosensitizers are photodynamic. This can be done by carrying out *in vivo* survival studies under strict anaerobic and aerobic conditions; if the photosensitization is photodynamic then there should be complete (or almost so) protection under anerobic conditions if the photosensitization is strictly photodynamic. As indicated previously there may be more than one photosensitizing mechanism in competition with each other, the absence of O_2 may favor an alternative mechanism which under aerobic conditions would not occur.

Once an O_2 requirement is established then the determination of Type I or Type II reaction can be conducted. The detection of $^1O_2$ *in vivo* can be accomplished by two main mechanisms: 1) Conduct survival experiments using D_2O instead of H_2O. (The D_2O solvent effect increases the $^1O_2$ lifetime ten fold, which also increases the sensitizers potency, Merkel *et al.*, 1972.); 2) Introduce known $^1O_2$ quenchers such as N_3 (azide) developed by Hasty & coworkers (1972). Other quenchers of singlet oxygen include: carotenoid pigments (Krinsky, 1968; Matheson & Rodgers, 1982); histidine (Matheson *et al.*, 1975); alpha-tocopherol (Ouannes & Wilson, 1968); bilirubin

(Matheson et al., 1974b); 2,5-dimethylfuran (DMFu) (Usui & Kamagawa, 1974); and DABCO (Ouannes & Wilson, 1968). The main problem with quenchers is that they do not always provide easily interpreted results. A particular quencher may be useful for one sensitizer and not another because it is not in proximity to the target to protect it or the sensitizer is too closely bound to the target and reacts before the quencher can react. Often the quenchers are themselves toxic at the concentrations needed to effectively quench $^1O_2$, (Ito, 1977). In addition to in vivo results, in vitro determination of $^1O_2$ production by sensitizers is useful (Ito, 1977). Some chemical traps for singlet oxygen which have been used in the past include: DMFu (Ito, 1977); 1,3-diphenylisobenzofuran (DPBF) (Matheson et al., 1974a; Lindig & Rodgers, 1981); 9,10-anthracene diproprionic acid (Lindig & Rodgers, 1981); and the bleaching of p-nitrosodimethylaniline in the presence of imidazole (Gommers et al., 1982).

To determine if Type I reactions are taking place producing O_2^- the addition of superoxide dismutase (SOD) to the in vivo system will scavenge any O_2^- present, (Michelson & Buckingham, 1974). The problems involved with any photodynamic photosensitization is that O_2^- can readily be converted to $^1O_2$ (the reverse is possible but less so) and other free-radicals such as $\cdot OH$ can also produce $^1O_2$ and O_2^- species

(Singh, 1978). This is further complicated by the fact that more than one reaction pathway may be involved in the photooxidation and this reaction scheme can change with changing reaction conditions (Spikes, 1977).

If oxygen is not involved in the reaction then an examination for other photochemical processes such as rates of photodegradation of the sensitizer, production of photodegradation products, and using various spectroscopic analyses of the excited state (MCD, ESR, UV) for the production of oxygen-independent free-radicals.

ix) Polyacetylene photosensitization:

Since the discovery of the phototoxic properties of polyacetylenes and their biosynthetically derived thiophene derivatives from the plant family Asteraceae (Camm et al., 1975; Chan et al., 1975; and Gommers & Geerlings, 1973), there have been reported apparently different in vivo modes of action for two of these plant secondary metabolites, the thiophene - alpha-terthienyl (Arnason et al., 1981a) and the polyacetylene - phenylheptatriyne (Arnason et al., 1980). Alpha-terthienyl was found to act as a typical Type II photodynamic sensitizer. Photosensitization of Escherichia coli B was present only under aerobic conditions but not under anaerobic conditions and exogenous

superoxide dismutase (SOD) had only a slight protective effect. The singlet oxygen quencher sodium azide, however, did protect yeast cells (Saccharomyces cerevisiae) from photosensitization. In contrast the photosentitization of E. coli cells by the polyacetylene PHT, was observed under both aerobic and anaerobic conditions. Sodium azide and SOD were not significantly protective in vivo.

Similar results were found for α-T and PHT photosensitized haemolysis of red blood cells (Wat et al., 1980). Haemolysis of red blood cells was enhanced under aerobic conditions when α-T was employed as a sensitizing agent, but no differences were seen with PHT under O_2 or N_2 conditions. The apparent lack of an oxygen requirement for PHT photosensitizations superfically resembles the mechanism of action with furanocoumarins. However, PHT does not form crosslinks in DNA (Wat et al., 1977) and has a minimal detectable mutagenic activity in sister chromatid exchange experiments using Chinese hamster cells (MacRae et al., 1980). Thus an alternative mode of action, perhaps via free-radical generation, has been proposed, (Arnason et al., 1980).

In order to determine whether this oxygen-independent photosensitization mechanism was a general phenomenon for acetylenic compounds versus their thiophene derivatives, a variety of compounds including thiophenes, polyacetylenes and mixed structures were examined for a number of parameters in this study. (See Figure 4 for structures

used in this study.) Differences in in vivo photosensitization under aerobic and anaerobic conditions were examined in detail for several compounds with two test organisms *E. coli* B & *S. cerevisiae* to determine the role of oxygen in photosensitization. In addition, relative phototoxicities with the above organisms were established for each of the compounds. The compounds were examined for their in vitro singlet oxygen generation capability using the $^1O_2$ chemical trap DMFu, their relative energy absorption of BLB radiation and their in vitro rate of photodegradation (which was thought to be indicative of the compound's ability to participate in a bond breaking/formation process). Since phototoxicity of compounds is also dependent upon the localization of the sensitizer in the cells as well as their photochemical capabilites, the relative hydrophobicity of the compounds was determined. An attempt was made to correlate relative toxicity of the compounds to their photochemical properties and hydrophobicity.

METHODS

i) Cell cultures:

In this study two cell lines were used: Eschericia coli strain B obtained from the School of Medicne, University of Ottawa and Saccharomyces cerivisiae strain XY 222-1A WT obtained from A.P. James, National Research Council of Canada. E. coli cultures were grown for 24 hr at 37°C to stationary phase in nutrient broth medium (Difco)(pH=7.0). S. cerivisiae cultures were grown to stationary phase in sabouraud dextrose broth (Difco)(pH=6.8), for 30 hr at 32°C. Cell counts and cell strain maintenance were carried out on the appropriate liquid media with 1.5% bacto-agar (Difco) added. Liquid cell cultures were initiated using a single colony from a stock cell line. Stock cell lines were maintained with bi-monthly transfer to new media plates.

ii) Monochromatic light source:

Nearly monochromatic light of appropriate wavelengths for excitation of polyacetylenes was obtained using interference filters with a 10nm half-bandpass width, (Corion Corporation). The transmission spectra of the filters used are presented in Appendix A. Intermediate

wavelengths of light could be obtained by placing the filter at an oblique angle in the light path resulting in a small wavelength shift, (eg. an 11° tilt from the axis perpendicular to the light source shifts the wavelength 10nm). The light source for the experiments was a 1000 watt high pressure xenon lamp unit (Orion Corp., Model 6410). A parallel light beam was obtained by focusing a concave mirror reflector and quartz lenses. Excess heat was removed from the light beam prior to the interference filter by a 10cm water cooled, water-filter. To avoid overheating, the interference filter was cooled in a high velocity air stream developed using a reversed vacuum cleaner system. The lamp housing was kept cool and ozone removed using a furnace fan vented into a fumehood. The exit light beam was directed downwards onto 5ml cylindrical quartz cuvette reaction vessels via a front surface mirror placed 45° to the light path. Exposure times were obtained using an electromagnetically controlled metal shutter apparatus between the interference filter housing and the 45° front surface mirror. The cuvettes each contained a small magnetic stirbar and were placed into holder recesses on a magnetic stir-plate to maintain constant agitation and uniform lighting of the sample. Light measurements were taken at the cuvette surface using a YSI radiometer connected to a strip-chart recorder for accurate measurements.

iii) Broad band near-UV light source:

Since polyacetylenes are exposed to broad-band ultraviolet solar radiation in nature, many experiments were conducted under a bank of 24 inch, 20 watt Westinghouse blacklight-blue (BLB) fluorescent lamps (FT20T12/BLB). These lamps provide near-UV radiation in the 300-400nm range with a peak emission at 350nm. (Solar and BLB emission spectra are shown in Appendix B.) The number of lamps and distance from the samples varied in some instances between major experimental series, each experimental design outlines the exact distance & number of lamps as well as the overall light output at the sample surface. To prevent sample overheating during an experiment the light bank was equipped with a small fan passing room air continuously over the samples.

iv) Polyacetylene compounds:

Eleven polyacetylene compounds were obtained from J. Lam, (Dept. of Chemistry, University of Aarhus, Aarhus, Denmark) and are listed in Figure 4. The purity of the compounds was checked by HPLC and UV spectra were taken upon their receipt on a Cary 219 spectrophotometer, (Appendix C). These spectra were used to recalculate the

Figure 4. Chemical structures of the polyacetylenes and thiophenes used in this study, including their reference numbers (used throughout this report), molecular weights, UV-A λmax's, & extinction coefficents.

Ref. #	Chemical structure	Mw	UV-A λ_{max}	$\epsilon_{UV\text{-}A\,\lambda_{max}}$
I	$CH_3-(C\equiv C)_2$-(thiophene)-$C\equiv C-CH(Cl)-CH_2-O-C(=O)-CH_3$	290·90	320	30,900
II	(thiophene)-(thiophene)-$C\equiv C-CH=CH_2$	216·33	343	21,600
III	(thiophene)-(thiophene)-(thiophene)	248·40	350	22,500
IV	$CH_3-CH=CH-C\equiv C-C$=(furanone)=O	160·17	347	21,200
V	(phenyl)-$(C\equiv C)_3-CH_3$	164·21	330	17,320
VI	(phenyl)-$(C\equiv C)_2-CH=CH-CH_2-O-C(=O)-CH_3$	208·27	299	32,400
VII	(phenyl)-$(C\equiv C)_2-CH=CH-CHO$	180·21	324	23,200
VIII	(phenyl)-$(C\equiv C)_2-CH=CH-CH_2OH$	182·22	297	17,800
IX	(phenyl)-$(C\equiv C)_2-CH=CH-CH_3$	166·23	299	22,650
X	$CH_3-CH=CH-(C\equiv C)_3-CH=CH-CH(Cl)-CH_2OH$	218·69	331	20,000
XI	$CH_3-(C\equiv C)_3-CH=CH-C(=O)-O-CH_3$	172·18	324	45,290
XII	$CH_3CH=CH-(C\equiv C)_2-CH=CH-C(=O)-O-CH_3$	174·20	308	13,100

concentrations of the stocks obtained from Lam. Periodically the concentrations of the stock solutions were checked for degradation using a Pye Unicam SP8-100 spectrophotometer.

In addition to Lam's compounds, phenylheptatriyne (PHT)(compound V, Figure 4) was extracted from Bidens pilosa leaves grown in a greenhouse, (University of Ottawa), (from seed collected from wild plants growing in Miami). The leaves were ground in a Waring blender with 95% ethanol (EtOH) to a slurry. This mixture was filtered using a Buchner funnel with Whatman #1 filter paper under vacuum to remove particulate matter. The resulting filtrate was partition-extracted three times with petroleum ether (P.E.) in a large separatory funnel after the addition of an equal volume of H_2O (1:1:2 EtOH:filtrate:H_2O:P.E.). The petroleum ether fraction which contained the polyacetylene had residual water removed by adding anhydrous sodium sulfite. After drying the P.E. fraction was reduced in volume on a rotary flash evaporator fitted with a continuous feed, (in vacu at 30°C). The resulting volume was placed at the head of a P.E. saturated silica gel column (100-200 mesh) and eluted with petroleum ether. Fractions were collected off the column and monitored for PHT using a Pye Unicam SP8-100 double-beam spectrophotometer. The fraction containing PHT ran before the first carotenoid band. It was reduced in volume on the rotary flash evaporator and crystals were

obtained by reducing the PHT fraction further using a nitrogen airstream to volatilize the petroleum ether and subsequently lowering the temperature of the remaining fraction to −25°C. The white needle-like crystals were collected by filtration using a fritted-disk glass funnel under vacuum and subsequently dissolved in ethanol. Purity was checked using gas chromatography, UV spectroscopy, mass spectrum, NMR spectroscopy, melting point, and high performance liquid chromatography (HPLC). Data shown in Appendix D.

v) Disk-test bioassay:

Sabouraud dextrose broth/agar plates were evenly streaked with a *S. cerevisiae* culture using a sterilized cotton tipped applicator. Polyacetylene compounds were applied in quadruplicate to 7mm Whatman #1 filter paper disks at a rate of 5 ug/disk. Two disks were applied while still slightly damp (to avoid polymerization of the polyacetylene) to each of 2 yeast streaked plates. One plate received 6 hr of near-UV light from two 18 inch 20 watt BLB fluorescent lamps, the other plate was kept in the dark. After the near-UV irradiation was completed both plates were incubated for 36 hr at 30°C. A semi-quantitative measure of phototoxicity was recorded as the distance cleared in mm about the disk on the UV

irradiated plate over and above any clearing seen on the plate maintained in the dark. Antibiotic activity was defined as clearing about the disk in the dark, which may or may not be near-UV enhanced. In addition to dark trials, filter paper disks containing only the carrier solvent (EtOH) were tested under light and dark conditions to ensure that there was no solvent toxicity.

vi) Aerobic versus anaerobic survival experiments:

The two test organisms used were E. coli and S. cerevisiae which were grown as described in section i). A cell pellet was isolated from approximately 30 ml of culture by centrifugation (5 min, 3000rpm) and was washed free of medium with sterile saline (0.87% NaCl) and reisolated in saline by centrifugation. Care was taken to maintain the culture under sterile conditions during the isolation proceedure. Stock solutions of cells were prepared in saline with optical densities of 0.3 for E. coli and 1.6 for S. cerevisiae at $n=10^7$ & 10^6 cells/1ml respectively.

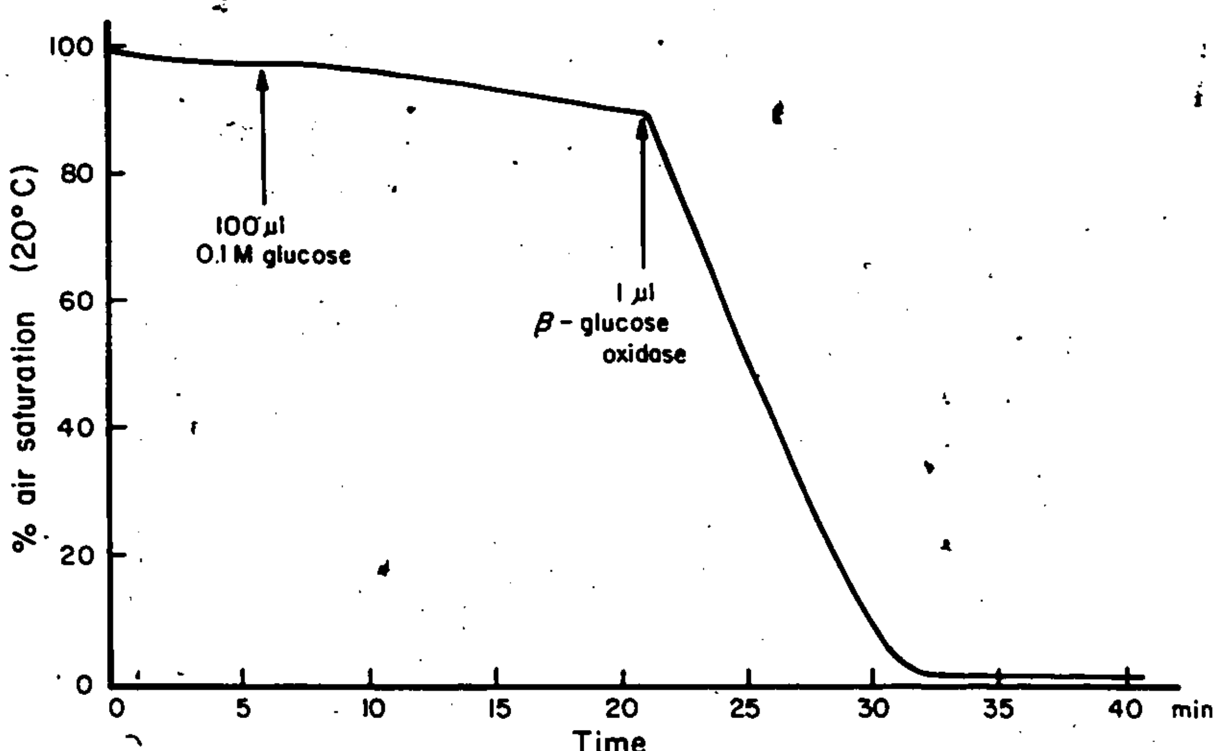
The reaction vessels were sterilized 5 ml cylindrical quartz cuvettes (pathlength 20mm) which were stoppered with gas-tight rubber septa. The cuvettes were filled with 2.5ml of the stock cell suspension with a hypodermic syringe. The cultures within the cuvettes were flushed

with sterile, humidified air or nitrogen ("Ultra-pure" O_2-free N_2, 99.99% purity) for 10 min to ensure that aerobic or anaerobic conditions were achieved respectively. The gases were humidified by passing them through a water-filled gas washing bottle and sterilized using a 0.22um membrane filter held in a polycarbonate syringe filter-holder fitted with a standard 22 gauge needle which punctured the cuvette septum. A second needle through the septum acted as a gas outlet. All needles were removed prior to the addition of the polyacetylene compounds to prevent volatilization of the polyacetylene out of the cuvette and to prevent oxygen contamination of the anaerobic trials. When a non-photodynamic response was obtained the trial was subsequently repeated using -glucose oxidase (Sigma) as an oxygen scavenger during irradiation. 0.5ul -glucose oxidase and 2ul 0.1M glucose were added to the cuvettes after gassing using a Hamilton 10ul syringe just prior to adding the polyacetylene. Figure 5 demonstrates the oxygen scavenging ability of this system on a fully aerated E. coli trial, using a Clark electrode to measure the oxygen tension.

The polyacetylene was added to achieve the appropriate concentration using a Hamilton 10ul syringe. The cuvette cultures were allowed to incubate in the dark for 10 min to allow uptake of the polyacetylene and O_2 scavenging by glucose oxidase when present.

At various time intervals during the UV irradiation of

Figure 5. Oxygen removal by β-glucose oxidase activity on an air saturated S. cerevisiae culture (5ml at 1.6 O.D.). Endogenous respiration recorded for the first 6 min in the absence of a carbon source (0.87% saline). At 6 min 100 ul of 0.1M glucose was added, and respiration monitored for an additional 15 min at which time 1 μl of glucose oxidase was added.


100
80
60
40
20
0
% air saturation (20° C)
100 µl
0.1 M glucose
1 µl
β - glucose
oxidase
0
5
10
15
20
25
30
35
40
min
Time

the trials 0.1ml samples were withdrawn using disposable sterile syringes fitted with short 25 gauge needles. The 0.1ml samples were added to 0.9ml sterile saline and subsequently serial diluted. 0.1ml aliquats of the serial dilutions were plated on the appropriate agar medium in triplicate and cell colonies counted after incubation to obtain survival curves.

Near-UV treated controls were initially carried out by adding equivalent amounts of the ethanol carrier solvent and irradiating. Polyacetylene treated trials maintained in the dark were carried out for each experiment. These dark trials were carried out in opaque 7ml vials with septa caps, and handled using the same procedures as outlined for UV irradiated trials.

In all trials cell cultures were constantly agitated using small teflon coated magnetic micro-spinbars within the reaction vessels to maintain uniform irradiation of the cell culture. Agitation during the dark incubation period with the polyacetylene present ensured a rapid and uniform uptake of the compound by the cells.

vii) Aerobic versus anaerobic photodegradation experiments:

S. cererivisiae was used for the determination and was grown in liquid culture as described in section i). Cells were harvested by centrifugation as described in

section vi), and a stock solution of cells with an O.D. of 0.6 prepared in 0.87% saline. Two 6ml samples of the cell stock solution were placed in test tubes sealed with septum caps and bubbled with air or N_2 for 10 min as described in section vi). To each of the two test tubes 2 ug/ml of PHT was added and allowed to incubate for 10 min to allow cell uptake of the polyacetylene before being placed in the cuvettes. Four cylindrical quartz cuvettes were used under the following conditions: 1) air + UV; 2) N_2 + UV; 3) air + dark; 4) N_2 + dark. To each cuvette 2.5 ml of the appropriate gas-saturated solution was added to gas-flushed, septum-stoppered cuvettes using a gas-flushed 5 ml glass syringe. Cuvettes and syringes were cleaned with hot dichromic acid and rinsed 7 times with distilled water prior to use to minimize loss of PHT to contaminated glassware. Ultraviolet trials were exposed to 1000 sec of monochromatic light at 310nm (10nm half-bandpass width), while the dark trials were maintained in the dark for the same period.

After UV irradiation the contents of each cuvette were added to a 10 ml centrifugation tube and centrifuged at 5,000 rpm for 5 min in a refrigerated centrifuge. The resulting supernatant was decanted into 1 x 0.3 x 4 cm microcuvettes. The resulting pellet from centifugation was washed once with saline and reisolated by centrifugation then lyse-extracted using 2.5 ml petroleum ether. This mixture was vortexed for 2 mins under reduced light

conditions, centrifuged and the petroleum ether supernatant placed in microcuvettes. UV spectra were taken on a Pye Unicam SP8-100 spectrophotometer.

Dark controls were used as initial concentration values, the light treated cell cultures determined the amount of PHT decomposed under anaerobic and aerobic conditions in the saline supernatant and within the cells as extracted by petroleum ether. Concentrations of PHT in ug/ml were calculated for the saline and petroleum ether fractions (extinction coefficients for PHT at $-_{max}$ 310 nm were 17,200 & 31,900 for saline and petroleum ether respectively). This experiment was done in triplicate.

viii) Relative toxicity bioassay:

The relative toxicities of the polyacetylenes were determined using the same organisms and apparatus as used in the aerobic-anaerobic photodynamic bioassay (section vi) under aerobic conditions only. Polyacetylene compounds were administered to give a final concentration of 10 ug/ml in the cuvette cultures. Samples (0.1 ml) were taken at time zero and after 10 min of irradiation from 2 blacklight-blue fluorescent lamps, then serial diluted and plated as described above to obtain cell counts. Experiments were carried out in triplicate.

ix) In vitro singlet oxygen generation studies:

Singlet oxygen gen[illegible] studies using the singlet oxygen chemical trap 2,5-dimethylfuran (DMFu), (Ito, 1978) were carried out at room temperature in air saturated glass distilled hexane (Caledon Laboratories Ltd.). The polyacetylene compounds and thiophene derivatives at 22.2 uM dissolved in hexane were placed in teflon stoppered 1 x 1 x 4 cm quartz cuvettes which served as irradiation chambers. After initial UV absorbance spectra were taken, 0.6 ul of DMFu (Aldrich) diluted 1:10 in hexane was added and the cuvette sealed against further air exchange. Ultraviolet illumination was provided by four unfiltered 24" (20 watt) BLB fluorescent tubes. Constant agitation during irradiation was maintained using a teflon coated magnetic micro-spinbar (7 x 2 mm) inside the cuvette. UV spectra were taken at regular intervals during irradiation using a Cary 219 spectrophotometer and the production of singlet oxygen was monitored as a function of the max absorbance decrease of DMFu (217nm), with three replicates. Singlet oxygen quantum yield for compounds III & V were carried out in EtOH in a similar manner to above, using monochromatic light at 350nm and 310nm respectively, in duplicate. Absorbance decrease of DFMu was monitored with a single beam Zeiss M4QIII spectrophotometer at 220nm.

x) Polyacetylene in vitro photodegradation studies:

In vitro degradation studies were carried out on a selected group of polyacetylenes in 50% EtOH in H_2O using broad-band near-UV light from 4 fluorescent BLB tubes. Polyactylenes were added to a final concentration of 22.2 uM in 1 x 1 x 4 cm teflon-stoppered quartz cuvettes. Constant agitation during irradiation was accomplished using a teflon-coated magnetic micro-stirbar (7 x 2 mm) inside the cuvette. UV spectra were taken at regular intervals during the irradiation using a Cary 219 spectrophotometer. Experiments were done at least twice for each compound. In vitro degradation was plotted as concentration versus irradiation time.

ix) Partition coefficient analysis:

Reverse-phase HPLC (high performance liquid chromatography) was used to determine the partition coefficients of the polyacetylenes used in this study following the methods of Konemann & coworkers (1979) and Ellgehausen & associates (1981). The HPLC system consisted of a dual pump system (Beckman 110A pumps) linked by a 420 Beckman Controller. Detection and UV absorption spectrum scanning was acheived with a 165 Beckman Variable Wavelength Detector. The column used was a Beckman

Ultrasphere 5μ ODS 4.6 mm x 25 cm (RP-C_{18}) and the solvent system was 70:30 methanol in water as used by Könemann & coworkers (1979). All compounds were dissolved in methanol to facilitate standardization of retention times. Detection was at 254nm.

A series of standard compounds with known water-octanol partition coefficients were chromatographed to establish a regression line for Log K' (capacity factor, defined in section vi Results) versus Log P (partition coefficient). Polyacetylene capacity factor (Log K') values were calculated from retention times.

RESULTS

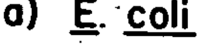
i) Aerobic versus anaerobic survival experiments:

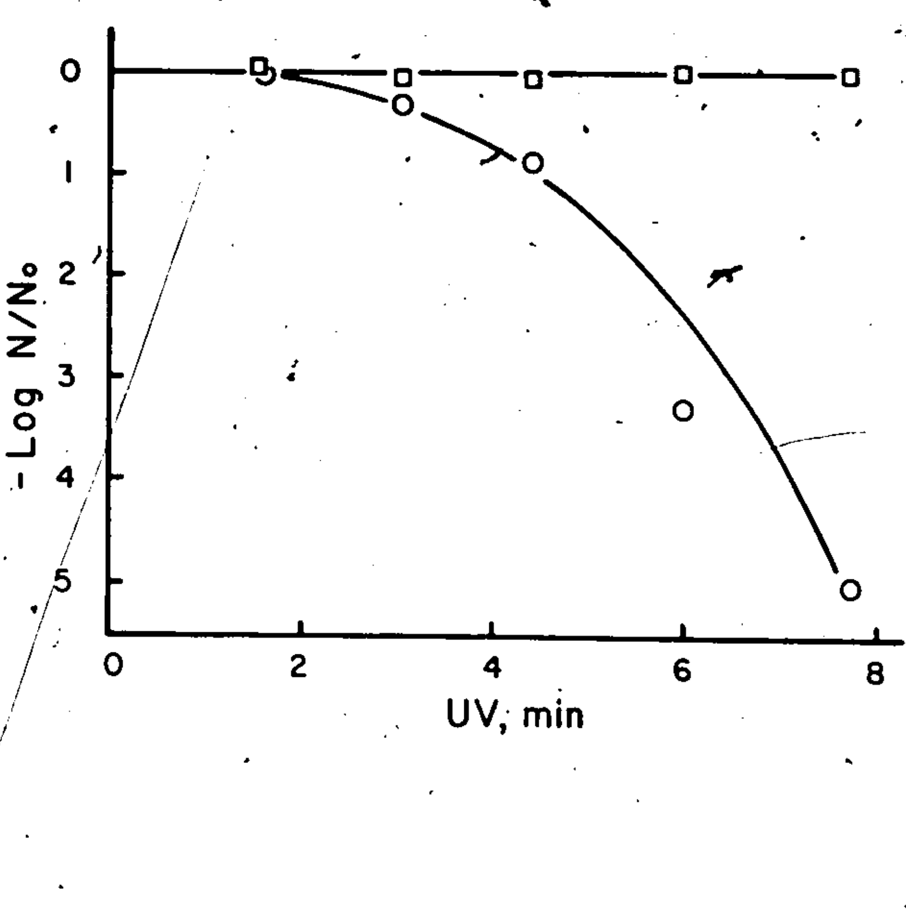
The most easily interpreted method for determining the presence of photodynamic activity for a photosensitizer has been to examine photosensitization under aerobic and anaerobic conditions. The thiophene alpha-terthienyl (α-T, compound III) was phototoxic under aerobic conditions but inactive under anaerobic conditions with both E. coli & S. cerevisiae as has been observed for many dye sensitizers such as methylene blue and rose bengal. This type of activity is characteristic of photodynamic action, (Figure 6, a & b).

A surprisingly different result was obtained with the straight chain polyaceylenes X & XII, which had similar light induced cell mortality under both aerobic and anaerobic conditions (Figure 7). This occurred for both E. coli and S. cerevisiae cells and was clear evidence for a non-photodynamic mechanism of action for these straight chain compounds.

Experiments with ring stabilized acetylenes under aerobic and anaerobic conditions indicated that both photodynamic and non-photodynamic mechanisms of action were possible for these substances. Initial experiments were carried out with PHT (compound V) and the prokaryote E. coli which had approximately equal mortality under both

Figure 6. Survival curves for E. coli and S. cerevisiae treated with the thiophene, α-terthienyl (III) and near UV radiation under aerobic (open circles) and anaerobic (open squares) conditions. Compound III at 1 μg/ml & 4 $w \cdot m^{-2}$ BLB in (a); and 1μg/ml & 4 $w \cdot m^{2}$ BLB in (b).





b) S. cerevisiae

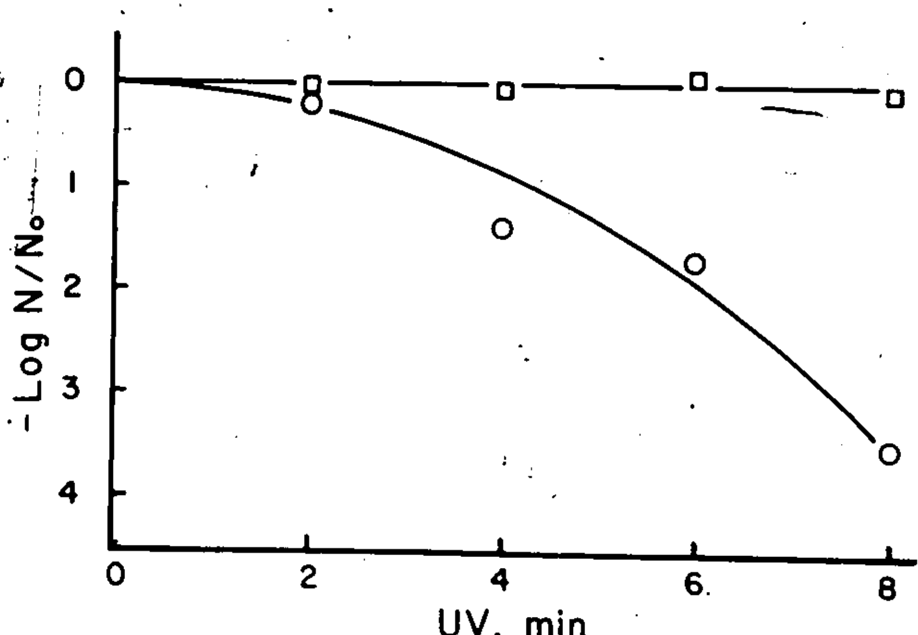
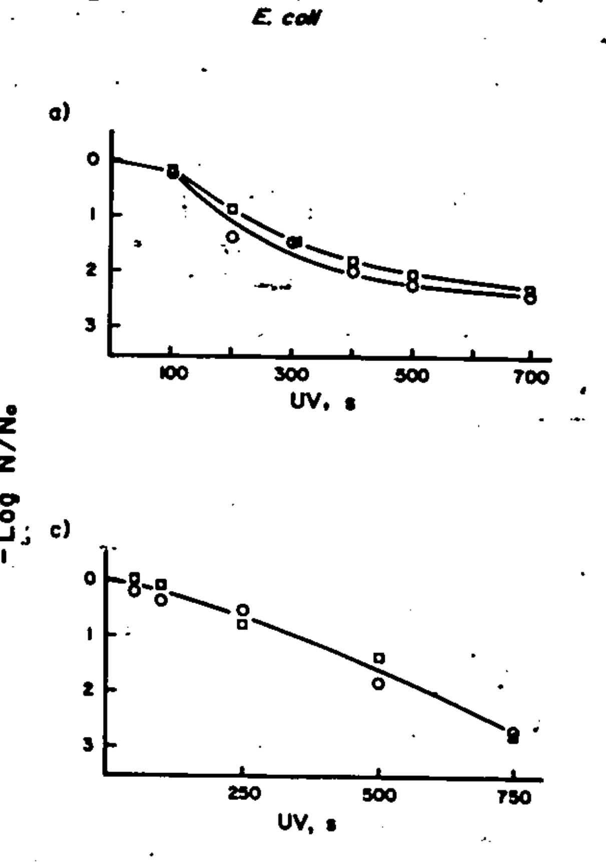
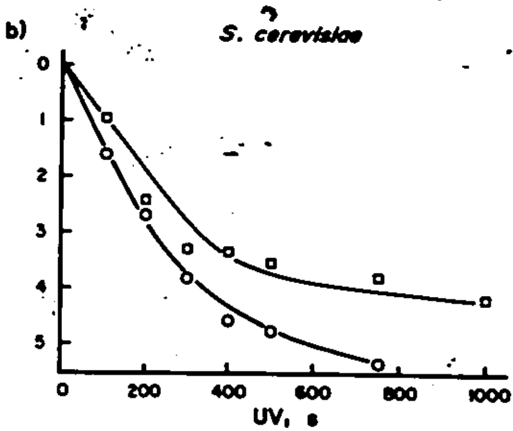
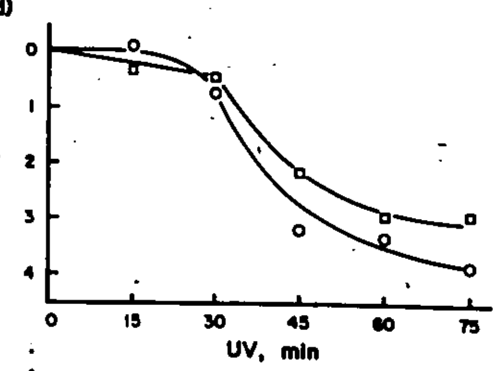


Figure 7. Survival curves for E. coli and S. cerevisiae treated with the straight chain polyacetylenes X, (a & b) or XI, (c & d) and near UV radiation under aerobic (open circles) and anaerobic (open squares) conditions. Compound X at 10 ug/ml, 4 wm^{-2} BLB for (a); 7.ug/ml, 4 wm^{-2} for (b); and compound XI at 4.ug/ml, 1.3 wm^{-2} monochromatic (346) for (c) & 1.ug/ml, 4 wm^{-2} BLB for (d).


E. coli
a)
-Log N/No
UV, s
c)
UV, s


b)
S. cerevisiae
UV, s

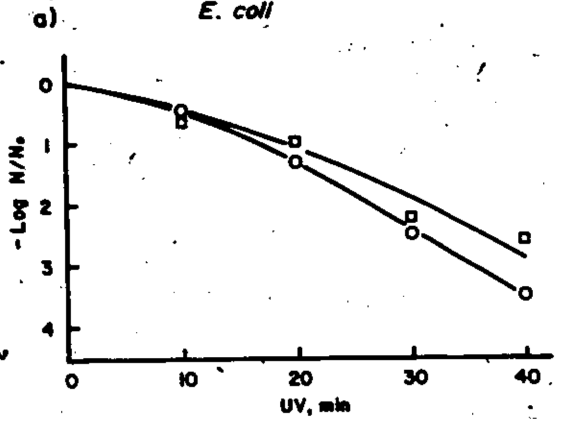

d)
UV, min

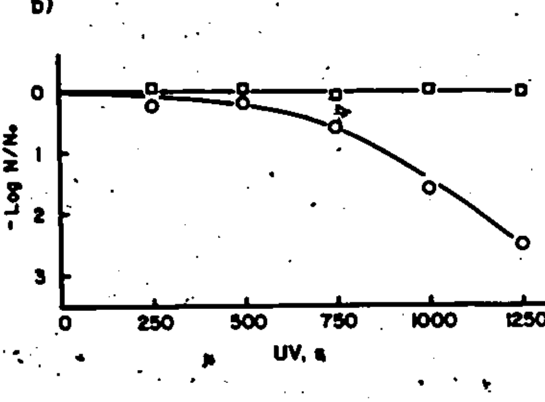
aerobic and anaerobic conditions (Figure 8a), indicative of a non-photodynamic MOA (mode of action). Subsequently PHT was treated against the eukaryote S. cerevisiae and unexpectedly the survival curves were indicative of a photodynamic MOA (Figure 8b). Further testing with another ring stabilized compound, VIII, had partial photodynamic activity with E. coli. Two of four trials had partial protection under anaerobic conditions, while the other two trials had considerably less protection under the same conditions as seen in Figure 8c. With S. cerevisiae as the test organism compound VIII clearly had photodynamic activity. Thus ring stabilized compounds appeared to show both photodynamic and non-photodynamic MOA's.

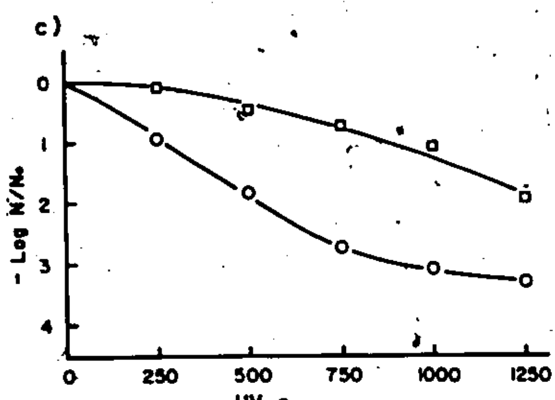
The acetate-phenylheptadiyne (VI) and the acetate-diyne-monothiophene-yne (I) were sufficiently phototoxic only to S. cerevisiae for further experimentation. Both compounds appear to have a photodynamic MOA as anaerobic conditions conferred protection from photosenitization (Figure 9 a & b). The lack of phototoxicity to E. coli is unexplained at this time, but it may be due to the presence of the acetate end-group.

In these assays each experiment was repeated at least three times and controls for the survival curves included cells treated with near-UV light and the small volume of ethanol used to carry the polyacetylene or thiophene, or cells treated with the compound but held in the dark. None of these treatments showed any significant effect on

Figure 8. Survival curves for E. coli and S. cerevisiae treated with the polyacetylenes PHT (V), a & b or PHD-ol (VIII), c & d and near UV radiation under aerobic (open circles) and anaerobic (open squares) conditions. Compound V at 1 ug/ml, 0.4 $w \cdot m^{-2}$ monochromatic light (λ_{310}) in (a) & 2 ug/ml, 0.4 $w \cdot m^{-2}$ monochromatic light (λ_{310}) in (b). Compound VIII at 10 ug/ml, 4 $w \cdot m^{-2}$ BLB in both (c) & (d).


a)
E. coli
-Log N/No
0
1
2
3
4
0
10
20
30
40
UV, min


S. cerevisiae
b)
-Log N/No
0
1
2
3
0
250
500
750
1000
1250
UV, s


c)
-Log N/No
0
1
2
3
4
0
250
500
750
1000
1250
UV, s

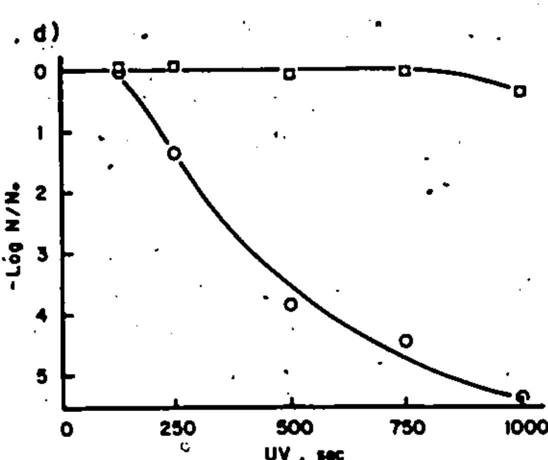
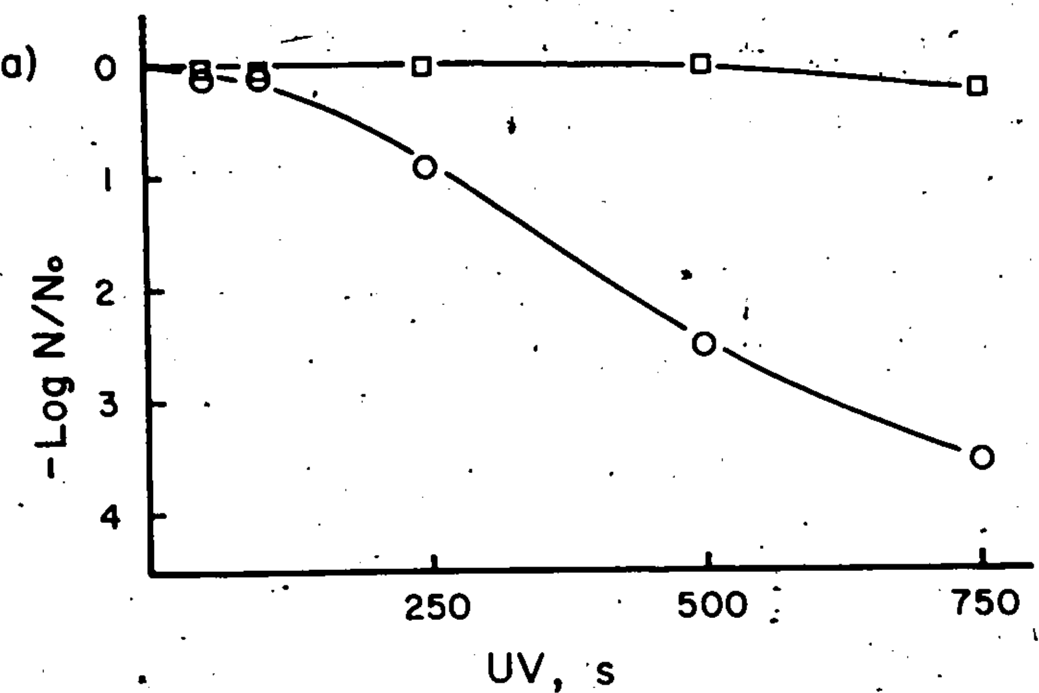
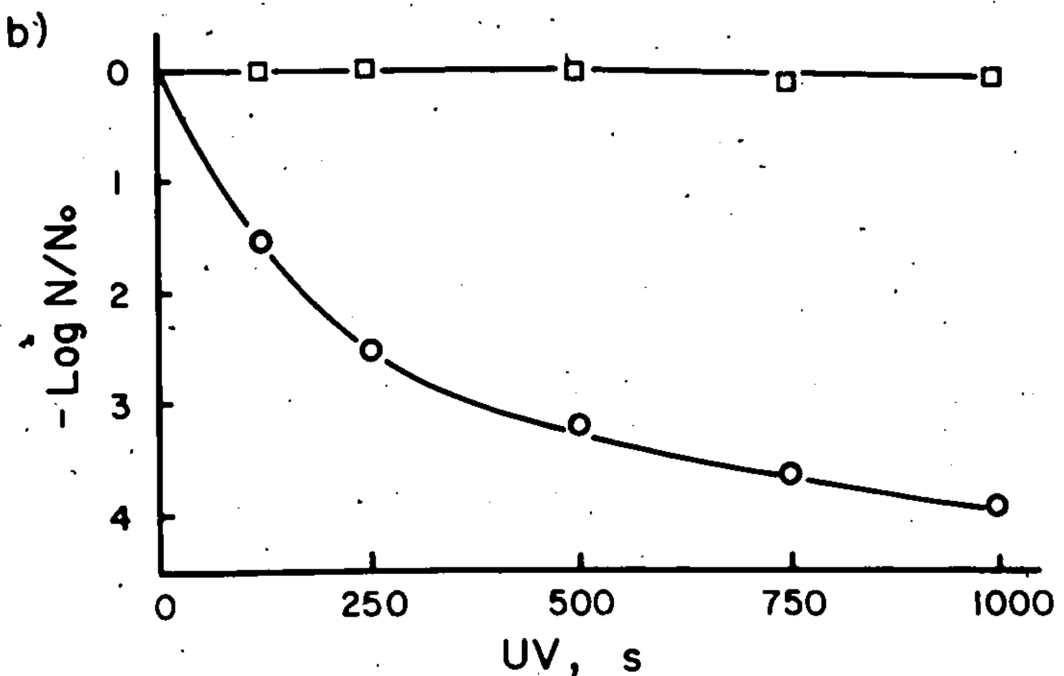

d)
-Log N/No
0
1
2
3
4
5
0
250
500
750
1000
UV, sec

Figure 9. Survival curves for S. cerevisiae treated with compounds, I, (a); & VII (b) and near UV radiation under aerobic (open circles) and anaerobic (open squares) conditions. Compound I at 3 μg/ml, 0.7 $w \cdot m^{-2}$ monochromatic light (λ_{320}); & VII at 10 μg/ml, 4 $w \cdot m^{-2}$ BLB.


a)
0
1
2
3
4
-Log N/No
250
500
750
UV, s


b)
0
1
2
3
4
-Log N/No
0
250
500
750
1000
UV, s

survival. In addition, a careful study of the anaerobic conditions indicated that control cells remained viable under N_2 conditions for periods longer than the treatment period used for these experiments.

In order to ensure that conditions were truly anoxic, additional experiments were undertaken with the addition of β-glucose oxidase & glucose to the anaerobic trial. Experiments with a Clark electrode indicated that these conditions were able to remove from a fully aerobic culture all oxygen detectable by the electrode within 10 minutes (see Figure 5, Methods). The glucose oxidase anaerobic trials yielded the same results as obtained with "ultra-pure" nitrogen purging alone.

ii) Aerobic versus anaerobic *in vivo* photodegradation:

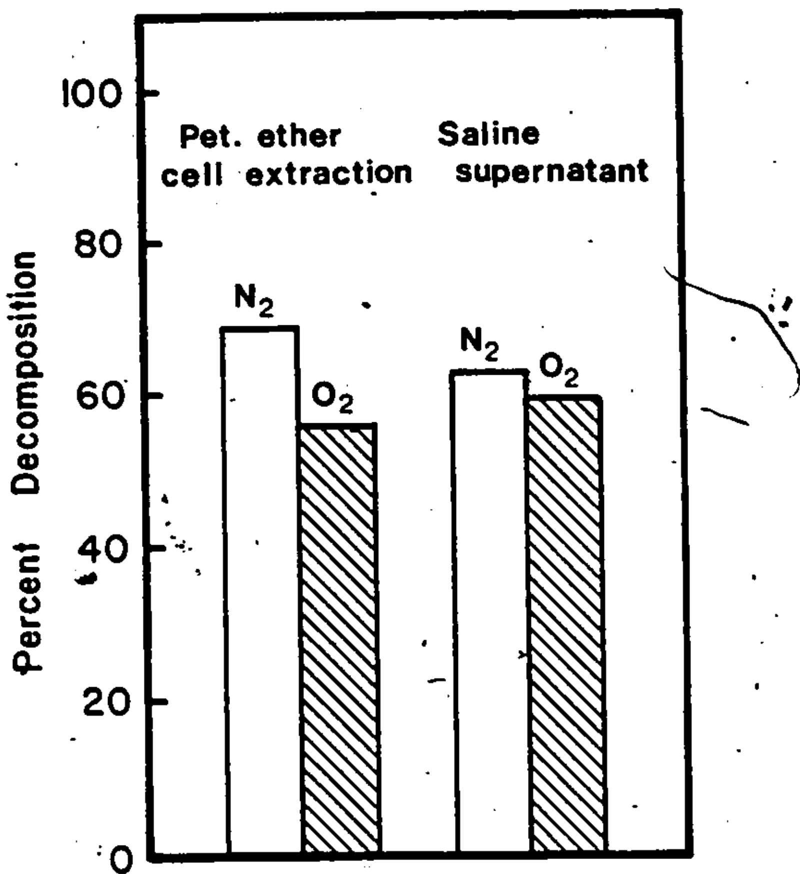
Polyacetylenes are known to be unstable (Arnason *et al.*, 1980) and one possible explanation for the apparent non-photodynamic effects could be a greater rate of disappearance of the photosensitizer in air as compared to N_2, thereby leaving a greater concentration of the photosensitizer in the N_2 trials. This was not found to be the case for PHT (compound V) in a saline solution by Arnason *et al.*, (1980) but a metabolic or other reaction within the cells was not considered. To test for differential decomposition within the cells a polyacetylene

(PHT) treated cell suspension was irradiated under aerobic and anaerobic conditions and the remaining polyacetylene estimated by centrifugation and solvent extraction. The results seen in Figure 10 clearly show that in both the supernatant and cell fraction there was a greater disappearance of PHT in N_2 treated samples. The lower disappearance of PHT under O_2 conditions suggests that energy transfer to O_2 is in competition with photodegradation processes. Thus differential disappearance of polyacetylenes is not a plausible explaination for the lack of an O_2 effect on cell survival.

iii) *In vitro* singlet oxygen generation:

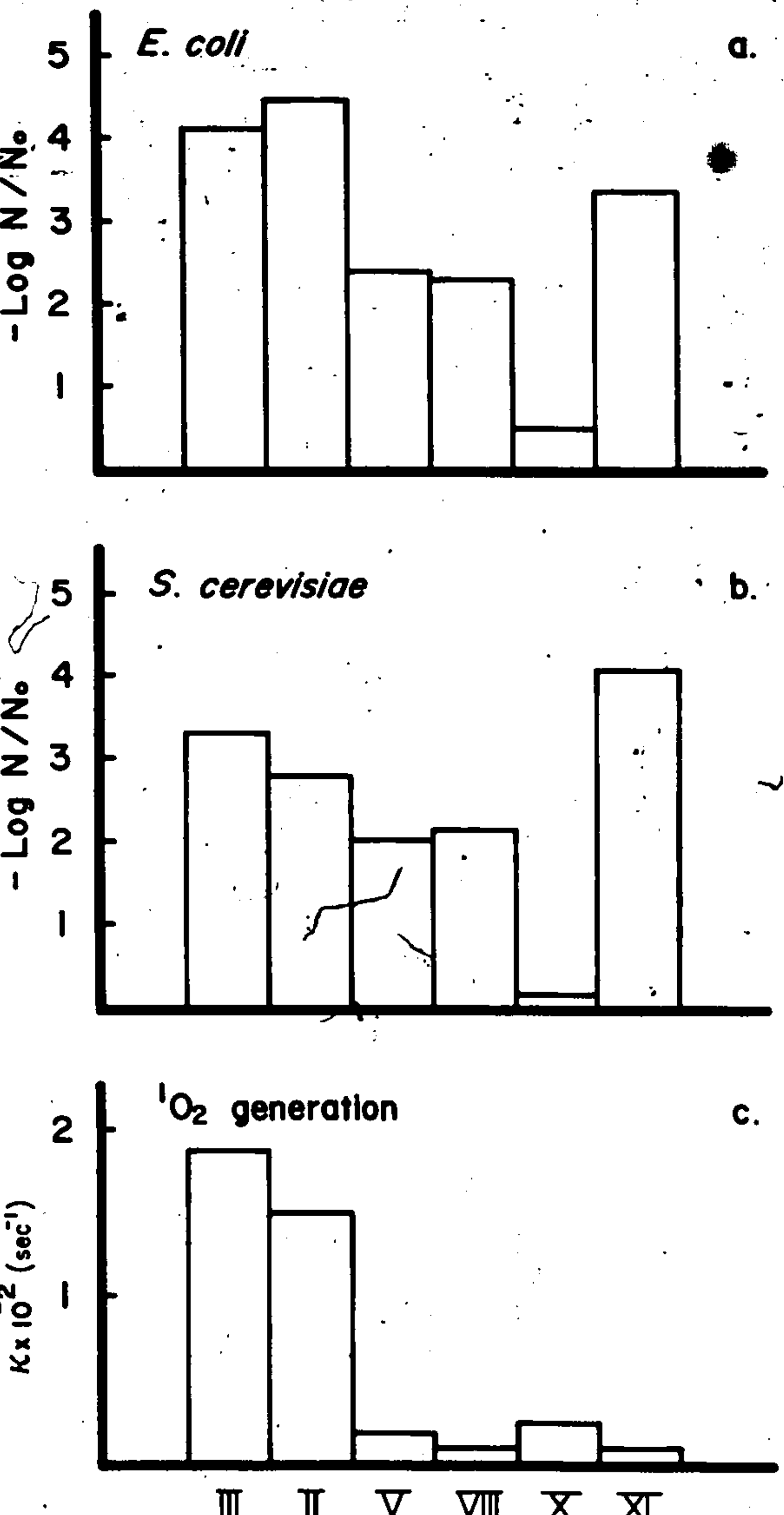
An alternative way of investigating the mechanism of action with respect to the oxygen effect is to examine *in vitro* $^1O_2$ generation using a chemical trap such as 2,5-dimethylfuran (DMFu)(Ito, 1978). The disappearance of DMFu, a function of singlet oxygen production, was found to be a pseudo- first order process for all compounds tested. First order rate constants were determined for singlet oxygen production from equimolar concentrations of all compounds using a broad band near-UV source (Figure 11). All of the compounds tested were found to be singlet oxygen generators under these conditions, but the

Figure 10. Percent decomposition of PHT added to a cell suspension of S. cerevisiae after light at 310 nm (0.6 $w \cdot m^{-2}$) for free and bound compound under aerobic and anaerobic conditions. Initial PHT concentration was 2 ppm.



100
80
60
40
20
0
Percent Decomposition
Pet. ether
cell extraction
Saline
supernatant
N_2
O_2
N_2
O_2

Figure 11. First order rate constants for *in vitro* singlet oxygen generation for equimolar concentration of compounds II, III, V, VIII, & X and near UV radiation from blacklight blue sources (4 $w \cdot m^{-2}$) compared to their relative phototoxicity to *E. coli* and *S. cerevisiae* under similar conditions.

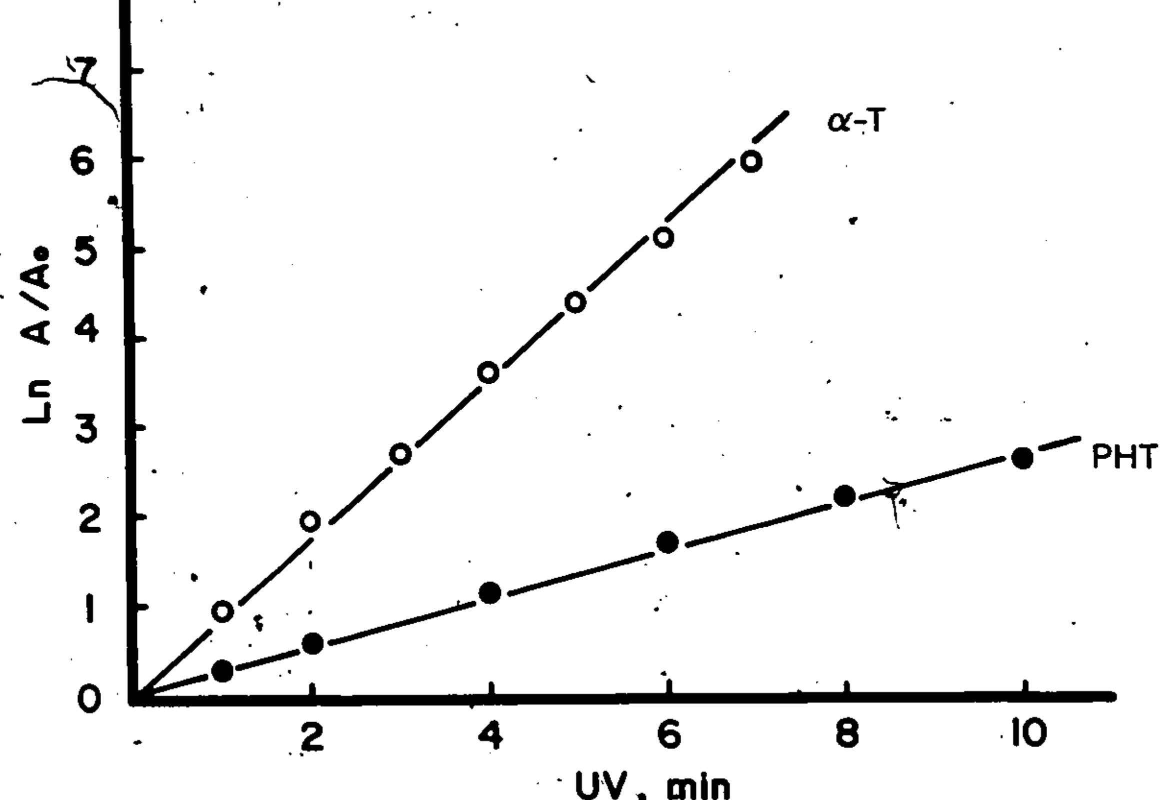

E. coli
a.
-Log N/No
5
4
3
2
1
S. cerevisiae
b.
-Log N/No
5
4
3
2
1
$^1O_2$ generation
c.
$K \times 10^{-2}$ (sec^{-1})
2
1
III
II
V
VIII
X
XI

thiophenes II & III were far more active than the polyacetylenes tested.

As seen in Figure 11 the photosensitization of yeast and E. coli by the polyacetylene and thiophene compounds is different from that which could be expected on the basis of singlet oxygen production alone. The polyacetylenes with the exception of X were far more important sensitizers than their $^1O_2$ capacity would suggest. This is a second line of evidence suggesting that a non-photodynamic mechanism may be operating with polyacetylenes. Other processes such as a number of site dependent phenomena may be important in explaining these results.

To determine if intrinsic molecular interactions with O_2 were responsible for the differences in $^1O_2$ generation, compounds III & V were examined for singlet oxygen generation on a quantum yield basis (Figure 12). For these experiments monochromatic light was used at the UV-A λ_{max} for each compound. When placed on a quantum yield basis, the yields of $^1O_2$ were similar for both compounds $\phi_T/\phi_{PHT} = 1.35$ using the equation ϕ is proportional to $(k/\lambda Ia)$ where k is the first order rate constant for $^1O_2$ production, Ia is the absorbed intensity as defined in Arnason et al., 1980. These results suggest that the efficiency of excitation transfer for III or V to oxygen is comparable. The lower singlet oxygen production of the polyacetylenes in broad band near-UV is therefore probably due to an

Figure 12. First order plot of the consumption of the singlet oxygen trap DMFu against the irradiation time using 4.4×10^{-5} M compound III (open circles) and V (closed circles). Monochromatic radiation at λ_{max} for III at 350 nm ($1.2\ w \cdot m^{-2}$) and V at 330 nm ($0.6\ w \cdot m^{-2}$) was used.


α-T
PHT
Ln A/Ao
UV, min
0
1
2
3
4
5
6
7
2
4
6
8
10

intrinsically larger absorbed photon flux in the thiophene compounds which have broader absorption bands and better overlap with BLB fluorescent illumination, since the extinction coefficients are similar for the two compounds.

iv) In vitro photodegradation studies:

As stated earlier polyacetylenes generally undergo relatively rapid photodegradation, which may be an important clue to their non-photodynamic toxicity as photodegradation does not require the presence of oxygen. Photodegradation can be envisioned as bond breaking/formation processes. During photosensitization polyacetylenes could undergo similar processes such as generation of toxic free radical species or photoaddition to membrane constituents (eg. unsaturated bonds in lipids). The photodegradation under broad band BLB fluorescent illumination of a number of thiophene and polyacetylene molecules was examined in vitro. The results (Figure 13) show that the kinetics of photodegradation are complex. The first order plots for thiophene degradation were linear suggesting first order kinetics while for the ring stabilized polyacetylenes V & VIII the second order plots were linear. In the above compounds the UV spectra merely collapse (Figure 14), while both of the straight chain

Figure 13. Rate of photodegradation of thiophene and polyacetylene compounds versus the irradiation time using near UV from blacklight blue sources (Intensity = 4 $w \cdot m^{-2}$). (For structures of compounds see Figure 4.)

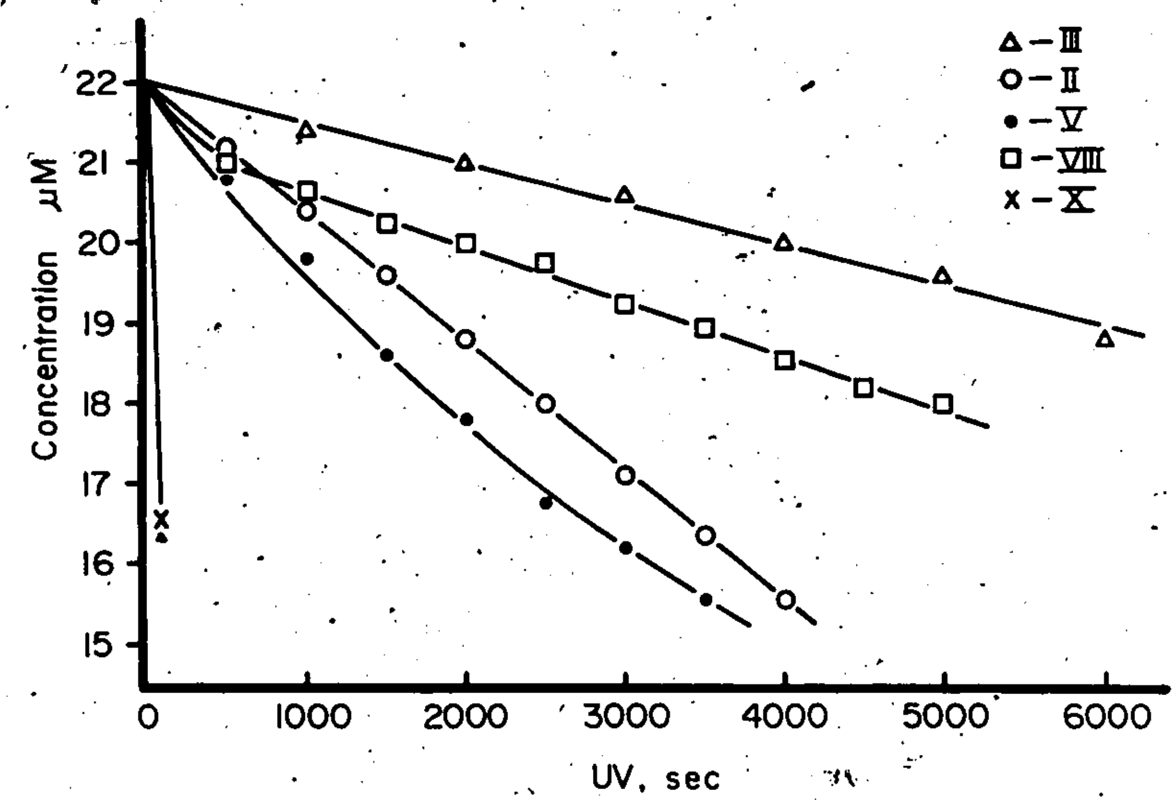
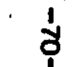

Concentration μM
22
21
20
19
18
17
16
15
0
1000
2000
3000
4000
5000
6000
UV, sec
△ – III
○ – II
● – V
□ – VIII
x – X

Figure 14. UV spectra from a time course study of the photodegradation of the ring stabilized polyacetylenes V & VIII, showing simple collapse of the spectrum. (Compound V photodegradation spectra were taken at 0, 500, 1000, 2000, 3000 & 4000 seconds and carried out in hexane. Compound VIII photodegradation spectra were taken as indicated.)



Compound V
- Photodegradation

Absorbance

2·0
1·5
1·0
0·5

210 230 250 270 290 310 330 350 370 390

Wavelength, nm

Compound VIII
- Photodegradation

Absorbance

1·5
1·0
0·5
·0

0
500
1000
1500
2000
3000
4000 seconds

210 230 250 270 290 310 330 350 370 390

Wavelength, nm

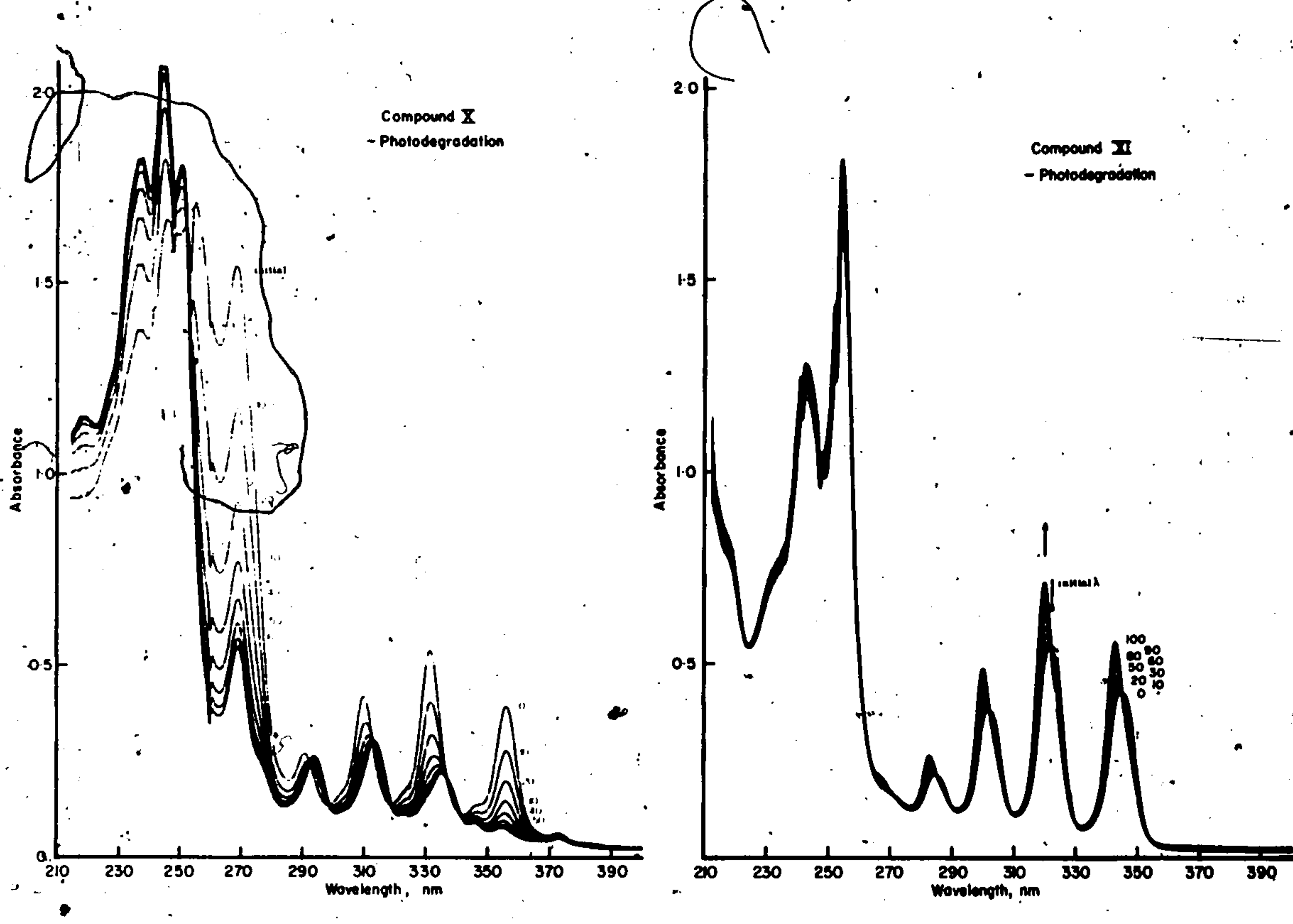
polyacetylenes X & XII underwent a very rapid initial photodegradation process from the parent compound to a product which degraded much slower than the parent compound (Figure 15). In compound XII it was impossible to resolve the parent and product UV spectra and this is therefore not reported in Figure 13, nor could an initial rate of photodegradation V_o be calculated.

The initial rates of photodegradation were calculated for five compounds seen in Table 2. The initial rates of degradation for the polyacetylene compounds were all higher than those of the thiophene III. All acetylenes except VIII have higher rates of degradation than the mixed thiophene-acetylene structure II. Rates of degradation were very high for the straight chain acetylenes and similar instabilities have been noted for several straight chain acetylenes that we have examined (data not shown). Therefore we might suggest that bond breaking/formation processes may play a greater role in polyacetylene photosensitization than for thiophene compounds.

v) Relative phototoxicity:

A total of twelve polyacetylenic structures and their derivatives were screened for phototoxicity using a semiquantitative disk-test to determine which compounds would be most useful for further experimentation.

Figure 15. UV spectra from a time course study of the photodegradation of the straight chain polyacetylenes X & XI, showing complex spectral colapse indicating the production of photoproducts. (Numbers beside spectra are irradiation times in seconds for each UV spectrum).



Compound X
- Photodegradation
Absorbance
Wavelength, nm
initial
Compound XI
- Photodegradation
initial λ
100
90
80
60
50
30
20
10
0

Table 2. Comparison of <u>in vitro</u> singlet oxygen production and photodegradation.

Compound	Singlet Oxygen Production	Photodegradation	
	k $(S^{-1})\times10^{-2}$	Vo $(\mu moles\cdot S^{-1})\times10^{-4}$	k/Vo $(\mu moles^{-1})$
II	1.5	17.8	8
III	1.8	5.6	31
V	0.17	26.6	0.64
VIII	0.084	15.5	0.54
X	0.084	580	0.014
XI	0.23	-	-

Photodegradation rates calculated from inital rates of photodegradation from Figure 13.

Phototoxicity and dark inhibition activity were expressed as the radius (mm) of the zone of inhibition and are tabulated in Table 3. The two thiophene compounds II & III had zones of inhibition generally much larger than the polyacetylenes. The larger zone of inhibition with these two thiophenes may be due to an inherently greater phototoxiciy. Alternatively the increased inhibition zone may have been due to the increased light stability of the thiophenes during long irradiation times as compared to polyacetylenes and/or an increased mobility of the thiophenes on the agar plates.

To establish a more accurate relative toxicity index it was necessary to eliminate some of these variables. This was accomplished by working in a liquid medium of a known cell density with a minimum of light interference from the medium and a relatively short irradiation time. This eliminated the diffusion problem and because irradiation times were short, photodegradation was less important.

The results of the liquid medium relative toxicity experiments) for E. coli & S. cerevisiae (Figure 16) did not follow the same pattern as seen in the disk-test bioassay (Table 3). The pattern differences seen in the two bioassays is probably due to the elimination of some of the variables inherent in the disk-test bioassay. There were also unexplained toxicity differences with some compounds between the two organisms.

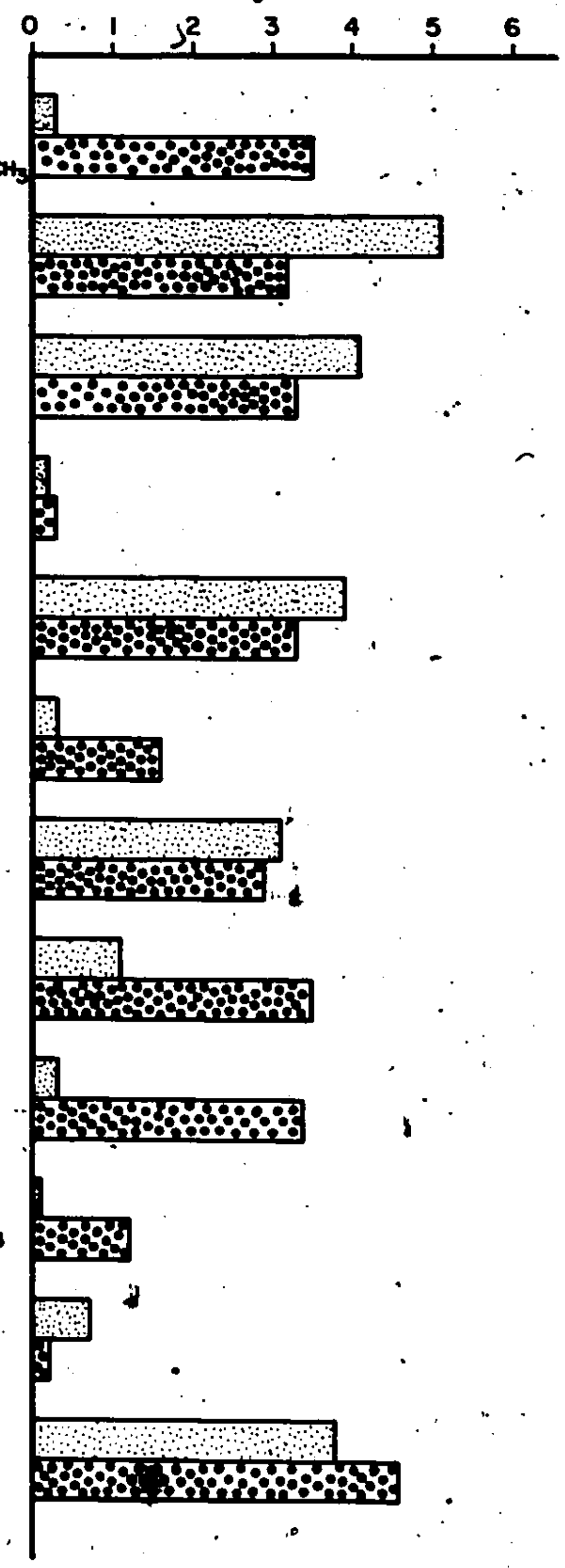
Table 3. Inhibition zones from compounds I through XII (excluding V) in a disk-test bioassay under dark and irradiated conditions (4 hr at 4 $w \cdot m^{-2}$ BLB). Compounds were applied to 7 mm filter paper disks at 5 ug/disk.

Compound	Zone of inhibition (mm)	
	+UV	-UV
I	4.3	0
II	37.5	0
III	22.0	0
IV	0.0	0
V	---	-
VI	1.5	0
VII	6.0	6.0
VIII	5.0	2.0
IX	1.1	0
X	13.8	2.0
XI	7.0	0
XII	6.5	0

Compound V not tested.

Figure 16. Relative phototoxicity for the compounds I through XII used in this study from the cell mortality of E. coli (small dot shading) & S. cerevisiae (large dot shading) in a liquid-medium bioassay. Compounds were administered at 10 μg/ml, and irradiated 10 for min at 4 $w \cdot m^{-2}$ BLB.

Relative toxicity



I $CH_3(C{\equiv}C)_2$–(thienyl)–$C{\equiv}C{-}CH(Cl){-}CH_2{-}O{-}C(=O){-}CH_3$

II (bithienyl)–$C{\equiv}C{-}CH{=}CH_2$

III (terthienyl)

IV $CH_3{-}CH_2{=}CH{-}C{\equiv}C{-}C(H){=}$(furanone)

V (phenyl)–$(C{\equiv}C)_3{-}CH_3$

IX (phenyl)–$(C{\equiv}C)_2{-}CH{=}CH{-}CH_3$

VIII (phenyl)–$(C{\equiv}C)_2{-}CH{=}CH{-}CH_2OH$

VII (phenyl)–$(C{\equiv}C)_2{-}CH{=}CH{-}CHO$

VI (phenyl)–$(C{\equiv}C)_2{-}CH{=}CH{-}CH_2{-}O{-}C(=O){-}CH_3$

XII $CH_3CH{=}CH(C{\equiv}C)_2{-}CH{=}CH{-}C(=O){-}O{-}CH_3$

XI $CH_3(C{\equiv}C)_3{-}CH{=}CH{-}C(=O){-}O{-}CH_3$

X $CH_3CH{=}CH(C{\equiv}C)_3{-}CH{=}CH{-}CH(Cl){-}CH_2OH$

The liquid medium relative toxicity results under broadband near-UV may suggest which compounds provide the best protective effect to the plant at the lowest metabolic cost. Thiophenes are generally among the most toxic, although the chlorinated straight chain molecule (X) had comparable toxicity. The ring stabilized acetylenes were moderately toxic. The straight chain acteylenes XII & XI and monoacetylene IV were least phototoxic. Some clear patterns of functional group chemistry appeared. For example acetates (I & VI) are much less toxic to E. coli than S. cerevisiae. The triacetylene V is more toxic than the structurally similar diacetylene IX. In the phenyl-diacetylene series the phototoxicity increases in the order IX, VIII, VI, VII on yeast and in the order IX = VI, VII, VIII on E. coli.

vi) Partition Coefficients by HPLC:

It has been suggested by other workers, in particular Ito (1977), that the particular localization of a photosensitizer within an organism can have profound effects on the MOA and phototoxiciy of a compound. The localization of a compound within the cell is largely governed by its partition coefficient, a measure of its lipophilicity (Hansch & Leo, 1979). To determine if partitioning of the polyacetylene in the cell correlated

with phototoxicity, partition coefficients were examined.

Partition coefficients were calculated from reverse-phase high performance liquid chromatography using the methods of Ellgehausen *et al.*, (1981). Standard compounds with known partition coefficients were chromatographed and a regression line established for partition coefficient (Log P) versus the capacity factor (Log K'), (Figure 17). The capacity factor K' is defined by the equation $K' = (t_R - t_o)/t_o$, where t_R is the retention time of the compound and t_o that of the non-absorbed chemical ie. methanol (Ellgehausen *et al.*, 1981). Polyacetylene partition coefficients were calculated from their capacity factors on the basis of the regression line (slope=3.03)(Figure 17 & Table 4). The analysis indicates that as a class the thiophenes are among the compounds that are most lipophilic. In addition the coefficient increases in the order of the mono-, bi-, and terthiophene. Acetylenes with the exception of IX & V are more polar than thiophenes. In the phenyldiacetylene series, the polarity increases as the end groups changed in the order methyl (IX), acetate (VI), aldehyde (VII), and alcohol (VIII). Increasing unsaturation slightly decreased the partition coefficient in two cases (IX as compared to XII)

Figure 18 compares relative phototoxicity to the estimated partition coefficients ($\overline{\text{Log P}}$). From these results it appears there is a weak but positive correlation

Figure 17. Partition coefficient (Log P) versus capacity factor (Log K') for a number of standard compounds with known Log P values. The capacity factors for the polyacetylenes used in this study are indicated with arrows on the best fit regression line (Slope = 3.03) of the standard compounds.

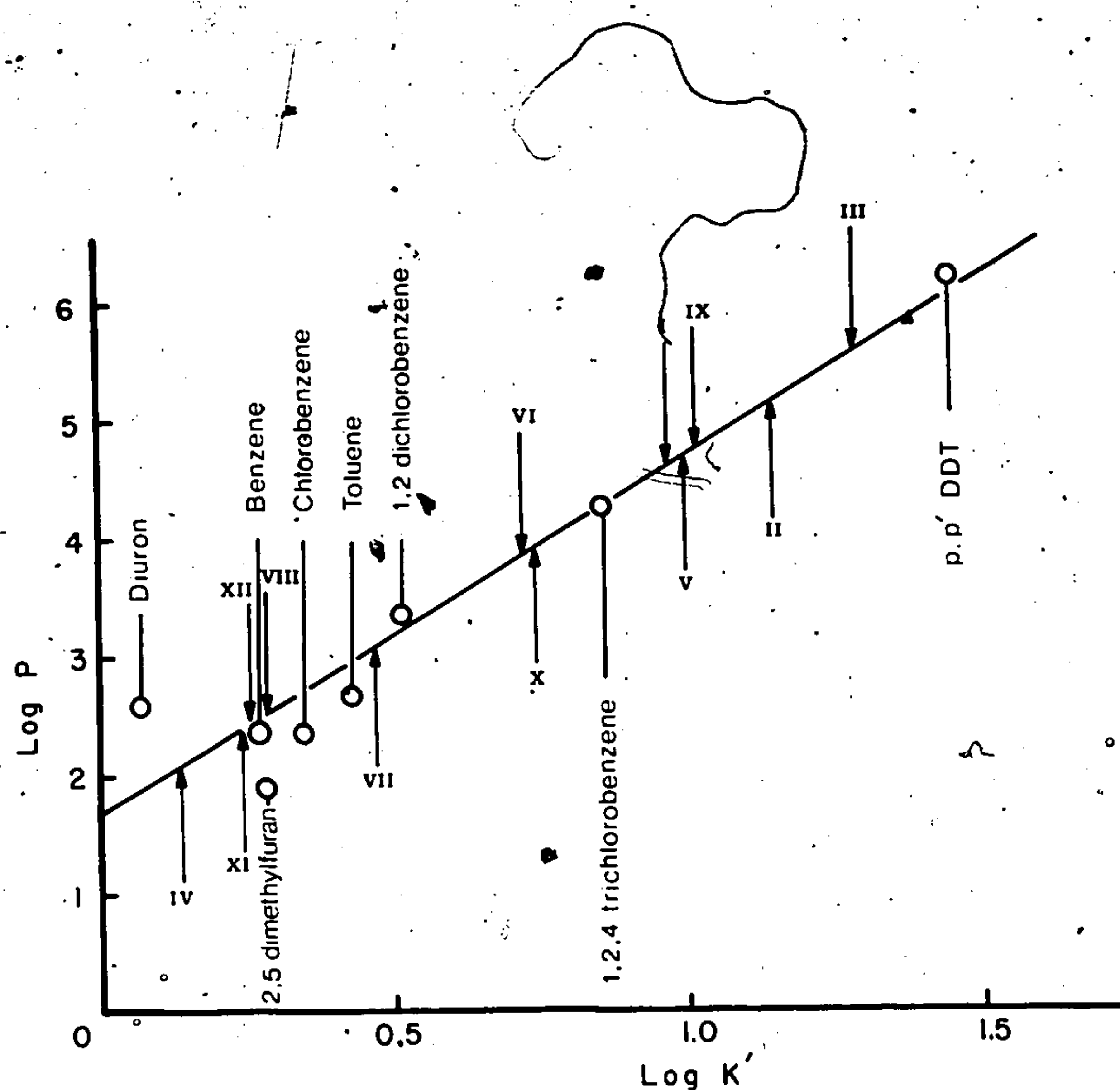
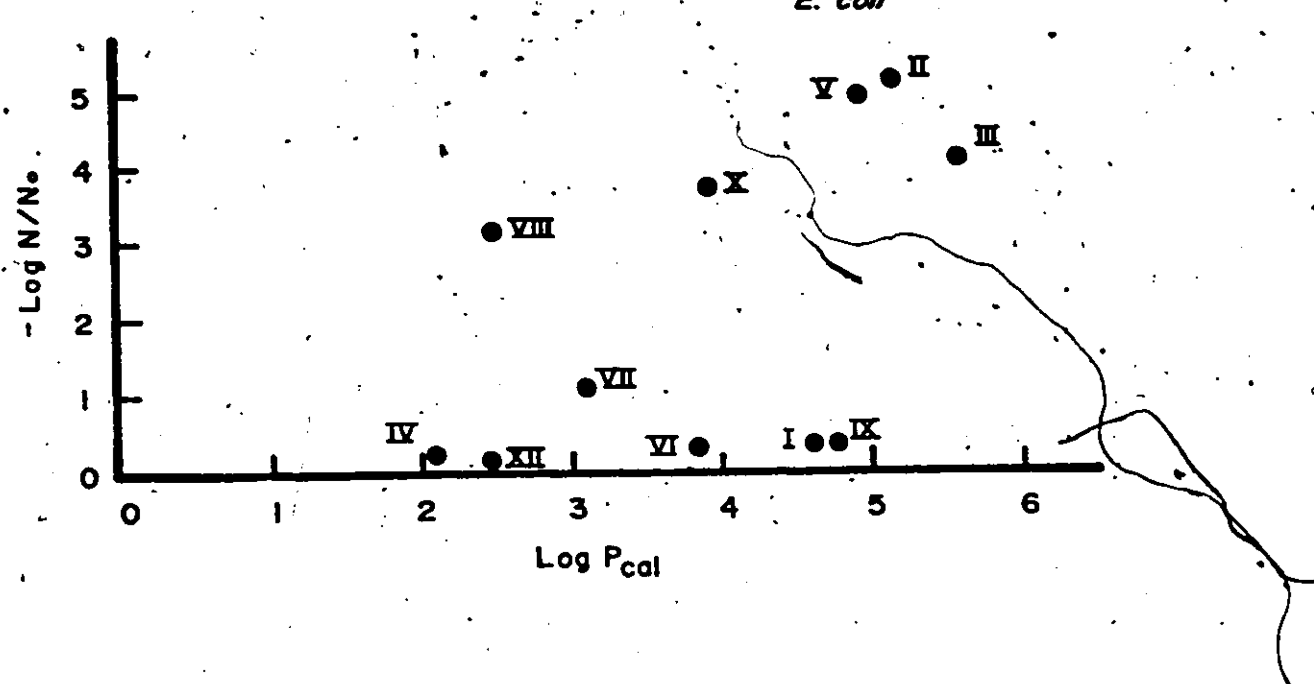

Log P
Log K'
Diuron
Benzene
Chlorobenzene
Toluene
1,2 dichlorobenzene
2,5 dimethylfuran
1,2,4 trichlorobenzene
p,p' DDT
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
0
0.5
1.0
1.5
1
2
3
4
5
6

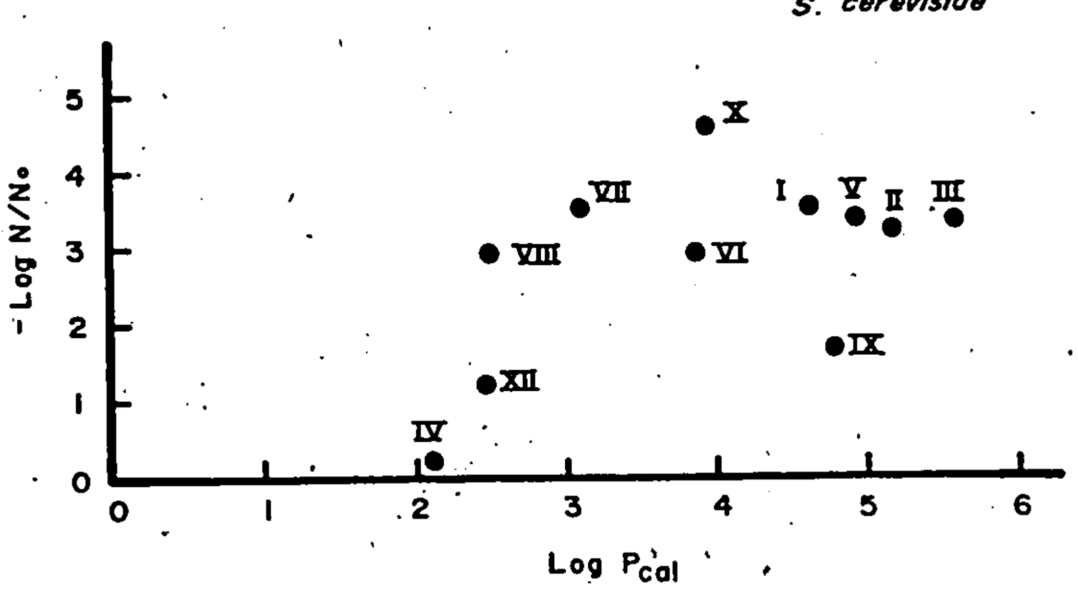
TABLE 4. Capacity factors (Log K') based on reverse-phase HPLC retention times and the calculated partition coefficient values (Log P_{cal}) from Log K' values for polyacetylenes used in this study.

Compound	Log K'	Log P_{cal}
I	0.97	4.60
II	1.15	5.15
III	1.29	5.57
IV	0.14	2.09
V	1.07	4.91
VI	0.73	3.88
VII	0.47	3.09
VIII	0.26	2.48
IX	1.02	4.76
X	0.74	3.91
XI	0.25 & 0.72	2.42 & 3.85*
XII	0.26	2.45

* cis & trans values.

Figure 18. Relative phototoxicity of the compounds from this study on E. coli and yeast compared to their respective estimated partition coefficients. Numbers beside points are the compound reference numbers (see Figure 4 for structures).


E. coli
- Log N/N₀
Log P_cal
0
1
2
3
4
5
6
I
II
III
IV
V
VI
VII
VIII
IX
X
XII


S. cerevisiae
- Log N/N₀
Log P_cal
0
1
2
3
4
5
6
I
II
III
IV
V
VI
VII
VIII
IX
X
XII

between phototoxicity and partition coefficient. The square of the correlation coefficient, r gives a measure of the amount of variation in phototoxicity which is explained by the partition coefficient. In the case of E. coli r^2= 0.264 (26%) and for S. cerevisiae r^2= 0.213 (21%).

In reference to specific compounds we found that thiophenes share the highest phototoxicity and partition coefficients, ring stabilized compounds have intermediate phototoxicity and partition coefficient values, and compounds IV and XII have the lowest phototoxicity and partition coefficients. The correlation does not always hold though, for example the acetates on E. coli and compound XI were less toxic than their partition coefficients would suggest. Also the slightly decreased parititition coefficient of more unsaturated compounds does not explain their increased phototoxicity, and in the phenyl-diacetylene series there was an inverse correlation between phototoxicity to E. coli and Log P values, but there was no correlation with yeast phototoxicities.

Relative Energy Absorption:

One possible explanation for the observed relative phototoxicities of the polyacetylenes in this study is the variation in energy absorption from the BLB source. This

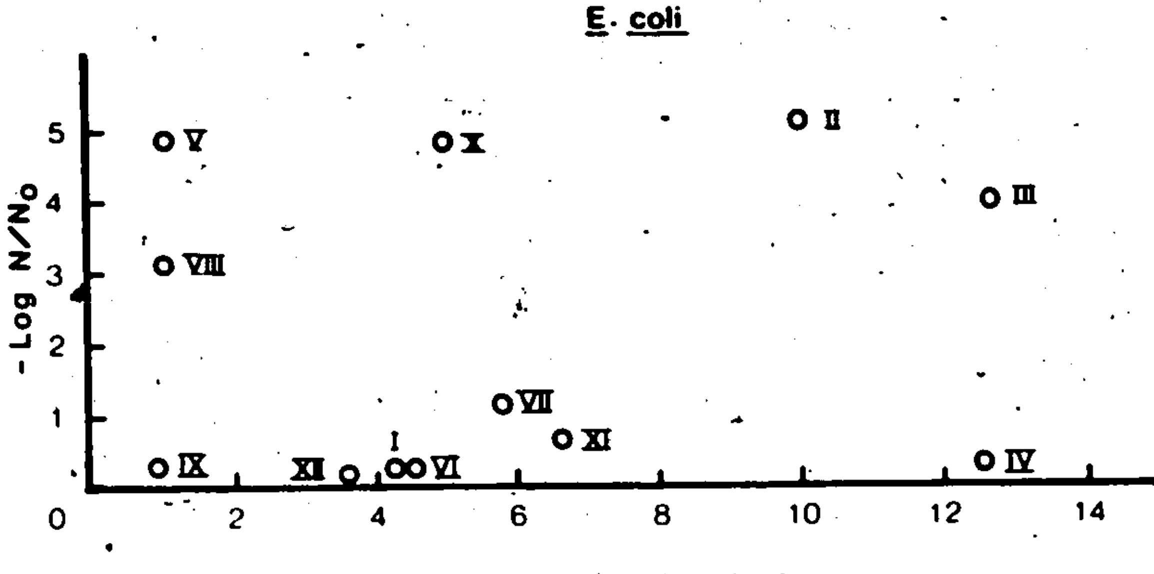
energy absorption can be estimated from the overlap of the UV spectrum of the compound and the relative energy emmission spectrum (Appendix B) of the BLB source defined by:

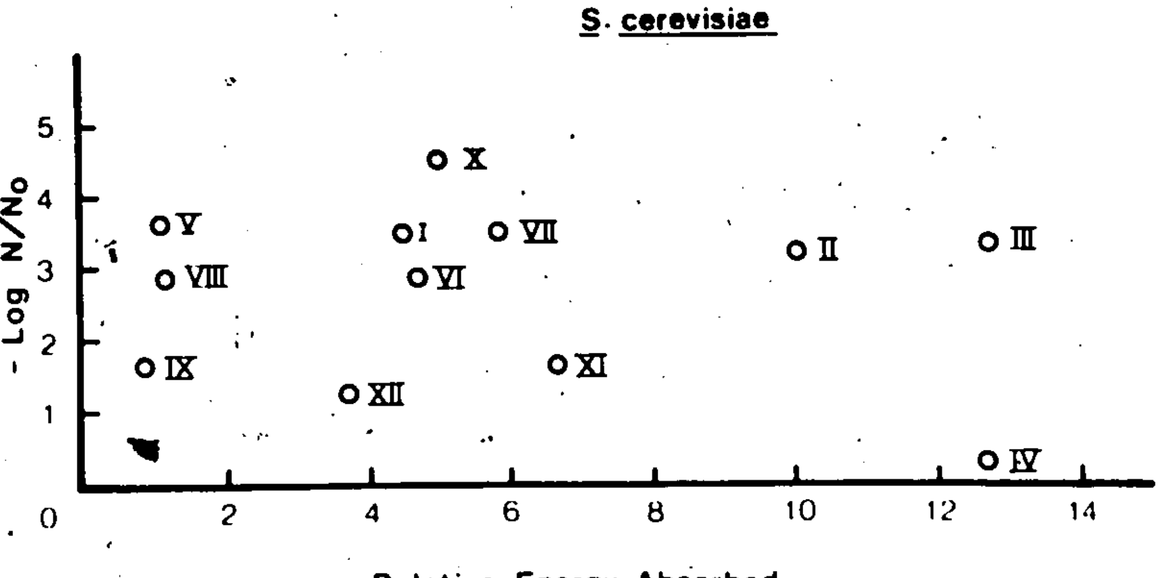
$$I_a(\lambda) = I_o(\lambda)\ (1-10^{-A(\lambda)}) \qquad 10$$

where $I_a(\lambda)$ is the absorbed intensity at wavelength (λ); $I_o(\lambda)$ is the incident intensity at wavelength (λ) from a BLB fluorescent source; and $A(\lambda)$ is the absorbance value of the compound at wavelength (λ). The intensity absorbed was calculated at 5 nm intervals, the total energy absorbed for the BLB source for each compound at the same concentration was integrated using the approximation of the area under the histogram of $I_a(\lambda)$ versus λ, as described in Arnason et al., 1980.

The results seen in Figure 19 clearly show that the thiophenes generally have a much greater energy absorption than do the polyacetylenes. The thiophenes also have relatively large phototoxicities. On the other hand the furan-polyacetylene compound (IV) has an equally large energy absorption, but a very low phototoxicity. The other polyacetylene structures have considerably lower energy absorbances and there is little or no correlation between energy absorbed & phototoxicity for either E. coli or S. cerevisiae (r=0.0830 & 0.1784 respectively). Although some polyacetylenes have relatively low energy

Figure 19. Relative phototoxicity (-Log N/No) versus relative energy absorption for the compounds from this study with BLB radiation against E. coli and S. cerevisiae (r = 0.083 & 0.178 respectively). Numbers beside points are the compound reference numbers (see Figure 4 for structures).


E. coli
-Log N/No
Relative Energy Absorbed
0
2
4
6
8
10
12
14
1
2
3
4
5
V
X
II
III
VIII
VII
XI
IX
XII
I
VI
IV


S. cerevisiae
-Log N/No
Relative Energy Absorbed
0
2
4
6
8
10
12
14
1
2
3
4
5
X
V
I
VII
II
III
VIII
VI
IX
XI
XII
IV

absorbances from BLB sources they can have as great or greater phototoxicity than do the thiophenes II &III. Possible explanations for this phenomenon might include: a) the presence of a more efficient phototoxic mechanism with some of the polyacetylenes relative to the thiophenes, b) the presence of a greater substrate/sensitizer affinity, and/or c) the availability of sensitive sites in the organism which are more susceptible to polyacetylene photosensitization mechanism(s) than membrane damage from thiophenes.

DISCUSSION

The present study establishes a new unique dualistic mechanism for polyacetylene sensitization involving the competition of photodynamic and non-photodynamic mechanisms and reconfirms the hypothesis that thiophenes are photodynamic. This study indicates that the relative phototoxicity of polyacetylenes & thiophenes is a complex function of many factors, dependent on partition coefficients of the substances, chemical structure and to a lesser extent relative energy absorbance.

i) Thiophene MOA:

The protective effect of anaerobic conditions using α-T as a sensitizer (Figure 6) agrees with the results of previous workers (Bakker _et al_., 1979; Arnason _et al_., 1981a, Downum _et al_., 1982; & Gommers _et al_., 1982), which clearly suggests that $^1O_2$ is responsible for membrane damage. This is further substantiated in this study by the high yield of $^1O_2$ produced _in vitro_ (Figure 11) and the fact that there is a relatively slow _in vitro_ photodegradation of α-T (Figure 13). Only one study has proposed a different MOA for thiophenes. Kagan _et al_., (1981) suggested that the MOA for α-T was non-photodynamic on the basis of N_2-O_2 experiments. However the

cells used in their anaerobic trials were grown (not merely treated as in our experiments or those of other workers) under anaerobic conditions which probably resulted in weaker cells than those grown under aerobic conditions and hence were more easily photosensitized or had a lower survival rate.

It has been suggested that α-T acts at the membrane level by Yamamoto & coworkers (1979) & Downum et al. (1982). The partition coefficient value calculated in this study (Table 4) is very high and suggests that α-T should partition and bioaccumulate in the hydrophobic core of the membrane. From their studies on dye sensitizers Valenzeno & Pooler (1982) suggest that in order for effective photosensitization to take place the sensitizer must enter into the low polarity hydrocarbon core of the membrane and still have a good triplet quantum yield. Such appears to be the case for α-T. Ito (1983) states that membrane components such as proteins, sterols & unsaturated fatty acids are very reactive and susceptible to singlet oxygen and free radical mechanisms. Furthermore he reports that lipid peroxidation in yeast cells seems to be a predominant process in membrane-attacking sensitizers. Lipid peroxidation is easily initiated by $^1O_2$ and once initiated proceeds in a free radical chain reaction which produces a large rigid region in an otherwise fluid membrane. The result of such a rigid region is membrane leakiness & subsequent cell lysis due to holes produced in

the membrane by sheer forces at the fluid-rigid interface. The presence of holes in membranes has been demonstrated by electron microscopy for α-T photosensitized erythrocytes reported by Wat et al., (1980).

The $^1O_2$ mechanism of action for α-T is also found in a number of other plant-produced sensitizers (see Table 5) as well as a great number of dye sensitizers. The $^1O_2$ MOA has long been known for the polynuclear quinones such as hypericin & the porphyrin sensitizers such as chlorophyll a (Structures seen in Figure 20). More recently the isoquinoline alkaloid berberine has been demonstrated as a photosensitizer producing $^1O_2$ by our group (Philogene et al., 1984). The common denominator among these compounds are their conjugated ring systems and the presence of a predominant fluorescence, (Spikes, 1977). The thiophene compounds also possess these qualities and as reported above also have a $^1O_2$ MOA.

ii) Polyacetylene MOA:

The results presented in this study represent three lines of evidence for a dualistic mechanism of action for polyacetylene compounds: 1) Aerobic versus anaerobic survival curves show that polyacetylenes can photosensitize cells in vivo in the absence of oxygen, in at least

Table 5. Phototoxic secondary plant substances: Chemical classes, metabolic pathways, & MOA's.

Phototoxic secondary plant substances	Chemical classification	Biosynthetic pathway	MOA
8-methoxypsoralen	linear furanocoumarin	shikimic acid	DNA crosslinking
Angelicin	angular furanocoumarin	shikimic acid	DNA monoadducts
Phenylheptatriyne	polyacetylene	fatty acid syn.	free radical addition
α-terthienyl	thiophene	fatty acid syn.	singlet oxygen gen.
Berberine	isoquinoline alkaloid	tryosine & phenylalamine	singlet oxygen gen.
Harmaline	β-carboline alkaloid	tryptophane	DNA monoadducts
Dictamnine	furanoquinoline alkaloid	tryptophane	DNA monoadducts
Hypericin	polynuclear quinone	condensed anthrones	singlet oxygen gen.
Encecalin	chromene	acetate	genotoxicity
Euparin	benzofuran	terpenoid	genotoxicity
Visnagin	furochromes	acetate	DNA monoadducts
Pisatin	isoflavanone	shikimic acid	free radical MOA
Chlorophyll a	porphyrin (Mg)	glycine & succinyl coenzyme A	singlet oxygen gen.

(Chemical structures seen in Figure 20).

Figure 20. Chemical structures of phototoxic secondary plant substances listed in Table 5. i) 8-methoxypsoralen; ii) angelicin; iii) phenylheptatriyne; iv) alpha-terthienyl; v) berberine; vi) harmaline; vii) dictamnine; viii) hypericin; ix) encecalin; x) euparin; xi) visnagin; xii) pisatin; & xiii) chlorophyll a.

i) OMe

ii)

iii) $C\equiv C-C\equiv C-C\equiv C-CH_3$

iv) S S S

v) $N^{\oplus}$ OMe OMe

vi) MeO N H N Me

vii) OCH_3 N O

viii) OH O OH H_3C OH H_3C OH OH O OH

ix) O MeO O

x) O HO O O

xi) OMe O O O H

xii) MeO O OH OCH_2O O

xiii) CH_2 CH CH_3 H_3C N N CH CH Mg H_3C N N CH_3 CH_2 R O CH_2 C=O O phytol

R= COOCH

some instances. 2) *In vitro* singlet oxygen studies show that polyacetylenes can produce $^1O_2$, but the quantity is not substantial enough to account for their observed relative toxicities. 3) Polyacetylenes undergo rapid photodegradation, which is suggestive that bond breaking/formation processes may participate in photosensitization processes as indicated by their low k'/V_o value (where k'= the rate constant for $^1O_2$ production and V_o the inital rate of photodegradation) as compared to the thiophene compounds II & III.

These results on polyacetylene-mediated photosensitizations indicate that on one hand there can be a triplet energy transfer process to ground state oxygen resulting in the formation of $^1O_2$, and on the other hand there are non-photodynamic processes which may involve photochemical bond breaking/formation. These two processes appear to be in competition for excited state energy, the distribution of which is possibly determined by a combination of chemical structure and the micro-environment of the sensitizer, i.e. localization of the sensitizer at the cell surface or imbedded in the lipophilic core of the membrane.

The polyacetylene photosensitizing MOA is much less defined and more complex than for their thiophene derivatives. This is due in part to the many types of structures and end-groups found in the polyacetylene class

and their inherently different multiple triple bond systems which are inherently different from the conjugated double-bond rings of the thiophenes.

The results presented here show that the O_2 dependency during photosensitization for polyacetylenes appears to be related to the structure of the compound and on the micro-environmental conditions associated with the sensitizer and the test-organism. This later observation is clearly demonstrated by the varied MOA for PHT against E. coli & S. cerevisiae (Figure 7). Previously Arnason et al. (1980) suggested a radical mechanism which this study corroborates with E. coli. However, it has been pointed out by Ito (1983) that sensitizer-substrate specificity is critical in radical mechanisms, which may explain in part the unexpected oxygen-dependency using the test-organism S. cerevisiae. Apparently the suspected lack of a proper sensitizer-substrate with S. cerevisiae has allowed the alternative $^1O_2$ MOA to dominate during this photosensitziation. The similarity in this study between the $^1O_2$ quantum yield for α-T & PHT clearly suggests that a singlet oxygen MOA is possible for PHT. Furthermore acetylenes are known to undergo $\pi \longrightarrow \pi^*$ transitions upon light absorption (Coulson, 1967) which are normally associated with type II photosensitizations. Recently, magnetic circular dichroism spectra of the excited states of polyacteylenes and thiophenes (McLachlan,

Arnason & Hollebone, unpublished results) has shown that polyacetylenes do possess strong triplet excited states. The fact that oxygen quenching is apparent in the _in vivo_ photodecomposition of PHT in this study (Figure 10) and the protective effect of O_2 on the _in vitro_ degradation of PHT found by Arnason _et al_. (1980) also suggests that $^1O_2$ and bond breaking/formation processes are in competition. In _E. coli_ apparently the bond breaking/formation processes of PHT are much more lethal than the $^1O_2$ produced, evident from the quenching effect of of O_2 on toxicity (Arnason et al., 1980).

The results of this study on compound VIII (PHD-ol) demonstrates that the alteration of the end group on the compound may result in a shift from one MOA to the other. This may be the result of a change in the actual photochemical properties of the polyacetylene or may be a reflection of a change in the sensitizer-substrate complex. It is difficult to delineate from this study which factors may be the most important. In this study VIII was much more variable in regards to O_2 dependency with _E. coli_ than were any of the other compounds or VIII on _S. cerevisiae_. This variability may be due to intrinsic differences in specific _E. coli_ cell cultures used in each trial such as differences in cell stage and physiology. These differences can affect photosensitization mechanisms (Ito, 1980), and might affect

the sensitizer-substrate complexes. On the other hand VIII was more stable than PHT (Figure 13) in the *in vitro* photodegradation studies which may enhance energy transfer processes to oxygen rather than bond breaking/formation processes. Wat and associates (1980) also found that PHD-ol was more stable than PHT in an NaCl-PO_4 buffer solution.

The alternative free radical mechanism has been demonstrated independently for some synthetic diacetylenes by several workers. Leaver & coworkers (1983) grew *Acholeplasma laidlawii* cells on C_{20} diacetylene compounds which were incorporated into the membrane lipid regions. When the cells were irradiated for 10 minutes with the UV-light from a low-power mineral lamp the cells turned a yellow colour (they were originally white). It was subsequently found that 70 - 97% of the diacetylene linkages were crosslinked according to the scheme in Figure 21. In 80% of the membrane's NADH oxidase activity was lost after 60 seconds of irraditation and 100% by 150 sec versus the loss of only 20% in cells grown on oleic acid. Lopez & associates (1982) working on photopolymers of diacetylenes in membrane bilayers found that the rate of formation was sensitive to the degree of argon purging (maximum rates of polymerization occurred with complete oxygen exclusion). Although the authors did not comment directly upon the quenching effects of oxygen on this photochemical process, it is evident that O_2 quenching

Figure 21. Schematic diagram depicting synthetic diacetylene polymerizations within artificial membranes after UV irradiation, (After Lopez et al., 1982).

is a competing reaction, such as reported in this study. Their results showed that polymerizable lipid diacetylenes could be incorporated into bilayer membranes and cell walls. They suggested that diacetylenes could be polymerizable even in the presence of membrane constituents other than lipids. Bhattacharjee & Patel (1981) found that some diacetylenes were almost inactive in that they did not polymerize while others were highly active and they suggest this is due to the micro-environment of the sensitizer. Similarly the results presented in this study are suggestive that the micro-environment of the sensitizer is and important factor during photosensitization reactions. The bio-membrane studies with synthetic acetylenic acids were undertaken to produce more stable artificial membranes. Hub and coworkers (1980) produced artificial liposomes with diacetylene membranes which were stabilized by photopolymerization. The resulting membranes were not destroyed by organic solvents nor by osmotic shock.

The photosensitization MOA for the straight chain compounds X & XI were both oxygen-independent with both *E. coli* & *S. cerevisiae* which is indicative of a dominant bond breaking/formation MOA (Figure 8). This is further substantiated by their very rapid *in vitro* photodegradation (Figure 13) and their relatively low *in vitro* $^1O_2$ production (Figure 11). In compound XI there is a very rapid *in vitro* photoisomerization within 60 seconds after which photodegradation proceeds at

a slower rate. Such photoisomerizations are not uncommon for some polyacetylenes and have been reported for cis-dehydromatricaria ester to the trans isomer (Sorenson et al., 1954); cis,trans-matricaria lactone (compound IV) to the all trans form (Lam, 1971) and $CH_3-CH=CH-(C\equiv C)_3-(CH=CH)-CH=CH_2$ to a trans-cis form (Kogiso et al , 1976). In the case of DME, its photoisomerization may reduce its phototoxic activity, especially in sensitizer-substrate complexes where photoisomerization processes may compete with phototoxic bond breaking/formation processes. This may possibly be the reason for the relatively low toxicity of DME on E. coli although at present there is no information on the effects associated with the sensitizer-membrane complex in regards to rates of photoisomerization in vivo nor the effects of in vivo photoisomerization on phototoxicity.

The results presented here clearly demonstrate the complexity of polyacetylene photosensitizations and the dualistic mechanism of action which is largely governed by the photochemical properties of each compound and specific micro-environmental conditions associated with the sensitizer-substrate complex. Recently Bakker and associates (1983) have proposed a similar bond breaking/formation process involving the photoactivaiton of some isoflavonoid phytoalexins (phaseollin; 3,6a,9-trihydroxypterocarpan; glyceollin; tuberosin and pisatin). Free radicals were detected using ESR

spectroscopy and the enzyme glucose-6-phosphate dehydrogenase was used as a bioassay for photosensitization using the oxygen quenchers N_3, BSA, histidine & methionine. In this system they detected strong ESR free radical signals and no involvement of $^1O_2$ in the bioassay. Further to this, irradiated pisatin in the presence of an in vitro $^1O_2$ chemical trap (olefin, adamatylidenadamantane) showed that no singlet oxygen was produced during photosensitizaton. It was suggested that these compounds could photoinactivate membrane bound enzymes via bond breaking/formation processes, similar to those suggested here for polyacetylene photosensitizations.

iii) Relative phototoxicity; structure-function relationships:

Examining Figure 16 there are some obvious structure-function correlations that can be made, although the reasons for these correlations may not be clear at present. One of the most striking similarities seen here is the effect of the terminal acetate group on the monothiophene (I) and phenylheptadiyne-acetate (VI) in regards to the apparent toxicity to S. cerevisiae but not to E. coli. Most of the other compounds have relatively similar toxicity to both organisms. The reason

for this differential toxicity with compounds I and VI is not clear from this study although the mechanism of action is apparently photodynamic on yeast (Figure 9). The capacity factors for these two compounds are not very similar (Log K'=0.97 & 0.73 for I & VI respectively) representing two orders of magnitude between their partition coefficients which probably limits the likelihood of these two compounds having exactly the same sensitizer-substrate complex. One hypothesis may be that E. coli recognizes the acetate group and metabolizes it or actively transports these substances into particular compartments for biochemical processing, while yeast cells do not and hence are sensitized.

The relative lack of phototoxicity for compounds XI & IV may be due to the fact that these compounds readily photoisomerize in vitro which may also be occurring in vivo. The photoisomerization reaction is probably competing with $^1O_2$ production and/or bond breaking/formation processes and may lead to reduced photosensitization. This does not follow though for compound X which is highly toxic but also undergoes photoisomerization.

More generally speaking, compounds with a high capacity factor (Log K') tended to have the greatest photosensitizing ability (Figure 18). The capacity factor appears to be related largely to the end group functionality and the overall molecular weight of the

compound and should reflect the localization of the sensitizer in the biological organism.

Although this study has a limited variation in the acetylenic bonds present, there is evidence from other workers that toxicity generally increases with increased acetylenic bonds per molecule (Towers et al., 1977 & Arnason et al., 1981b). This generally holds true for polyacetylenes presented here with some exceptions such as XI as mentioned above.

It was suspected that the amount of energy available to a compound would correlate with its relative phototoxicity. The present study suggests that this is not necessarily true. Although the thiophenes do have relatively high phototoxicities and have good band overlap with BLB fluorescent radiation, some polyacetylenes such as compounds V & X have relatively low energy absorption values, yet are as toxic as the thiophenes. The similarity in the levels of toxicity in this case suggests that the thiophenes and at least some of the polyacetylenes operate via different photosensitization mechanisms. On the other hand the polyacetylene IV which had band overlap with the BLB source similar to that of the thiophenes had a very low relative toxiciy. Therefore the amount of band overlap appears not to be the major determining factor in polyacetylene photosensitizations.

Other factors to consider in relative toxicity are of course related to the photochemical characteristics of the

particular compounds. Clearly from the relatively small sample size of six compounds (Figure 11 and Table 2) the ability of these molecules to participate in one or both proposed MOA's above also determines the relative toxicity in a general way, but a more extensive examination using a greater number of polyacetylenes is necessary before this question can be answered definitively.

iv) Convergent evolution of phototoxicity:

From Table 5 it is seen that many different classes of plant secondary products have phototoxic properties and as well have utilized a diversity of phototoxic mechanisms. The mechanisms can be generally classified into: photodynamic, DNA photobinding, and free radical. These phototoxic mechanisms have arisen independently in quite different biosynthetic pathways. Hence, this convergence in evolution suggests there must be a strong selective advantage for phototoxic chemical defense. The most evident advantage is the acquisition of excited state chemistry over thermal or enzymatic chemistry. Excited state chemistry leads to a greater diversity of chemical reactions whch occur spontaneously once the sensitizing molecule has been excited by light. In the case of plants, light is a relatively free commodity which plants already use in photosynthesis.

The efficiency of the protective effects conferred on plants with phototoxic SPS's is evident from the fact that often these phototoxic substances are found in the plants at very low levels. As an example polyacetylenes are often found in Asteraceae species at 0.1% or less fresh weight and can effectively arrest larval development at that level (McLachlan _et al_, 1982 & Champagne _et al_, 1983). Alternatively tannins which are not phototoxic, must be found at 3 to 5% Levels in the plant to arrest larval developments (Feeny, 1970). Thus there may be a lower energy cost in maintaining phototoxic defense compounds than conventional non-phototoxic secondary plant substances.

There is some evidence that the phototoxic defense mechanism is more advanced relative to other chemical defense mechanisms as phototoxicity is generally found in the most advanced phylogenetic groups at the family, genera & species levels. Associated with the elaboration of phototoxic substances is often a reduction in the production of other secondary substances. The reduction in the types of chemical defense substances produced by a plant is generally considered an advanced character (Sorenson, 1977). This is evident in the advanced genus, _Tagetes_ of the Asteraceae. This genus elaborates thiophene compounds almost exclusively and are otherwise chemically depauperate (Sorenson, 1977) of the normal defense compounds found in most other members of the

Asteraceae, eg. sesquiterpene lactones. The thiophene compounds though are highly phototoxic and very efficient producers of $^1O_2$, thus the plant minimizes the metabolic cost of chemical defense with maximum results.

The use of excited state oxygen molecules is not limited to plants nor to photochemical reactions. Recently Babior & Parkinson, (1982) have shown that human NK lymphocytes produce superoxide radicals enzymatically through a high energy reaction, which are used to kill pathogens. This similarity in animal and plant defense is a second example of the convergent evolution process, this time occurring at the kingdom level.

v) Future Experiments:

From the experiments presented here several further experiments could be carried out to elucidate the MOA of polyacetylenes.. As presented here the photoaddition of polyacetylenes to membranes or another radical mechanism seems a probable mode of action. The use of a liposomic membrane system of a known composition to elucidate the radical process is an obvious first step. The establishment of the photoaddition process and its products is primary to confirming the MOA for polyacetylenes. The examination of a series of polyacetylenes with different end-groups could more clearly demonstrate structure-function relationships than is possible in the

present study.

In addition to determining the photoaddition products using liposomes, ESR spectroscopy could possibly establish direct evidence for the presence of free radicals in vivo, similar to experiments carried out by Bakker et al., (1983). ESR studies could be used to establish the competition between the two proposed MOA's for polyacetylenes. These results are important in that the decay rate of the free radical ESR signal may be important in the competition between the MOA's. For example it should be possible to establish directly O_2 quenching of a free radical ESR signal produced under anaerobic conditions.

Since much of the evidence presented here is from indirect in vitro studies and as such the actual sites of action in the membrane and the physiological changes which polyacetylene photosensitizations elicit in vivo has not been established. More direct results on the MOA for polyacetylenes and sites of action may be obtained from detailed studies on cell physiological changes during photosensitization. The examination of transport, membrane potentials and K^+ ion leakage could be valuable studies in elucidating the MOA's for various polyacetylenes and their thiophene derivatives.

A further study which may be of some significance could be the examination of membrane constituents in E. coli & S. cerevisiae before and after photosensitization.

This could be especially useful where radiolabelled polyacetylenes were used to establish if and where photoaddition products occurred in cells, and to be able to deal with structure-function relationships more directly.

Since this study has also shown that photoisomerization is possible for at least some polyacetylenes, a detailed examination of this process *in vivo* in regards to photosensitization may be useful in our further understanding of polyacteylene photosensitization.

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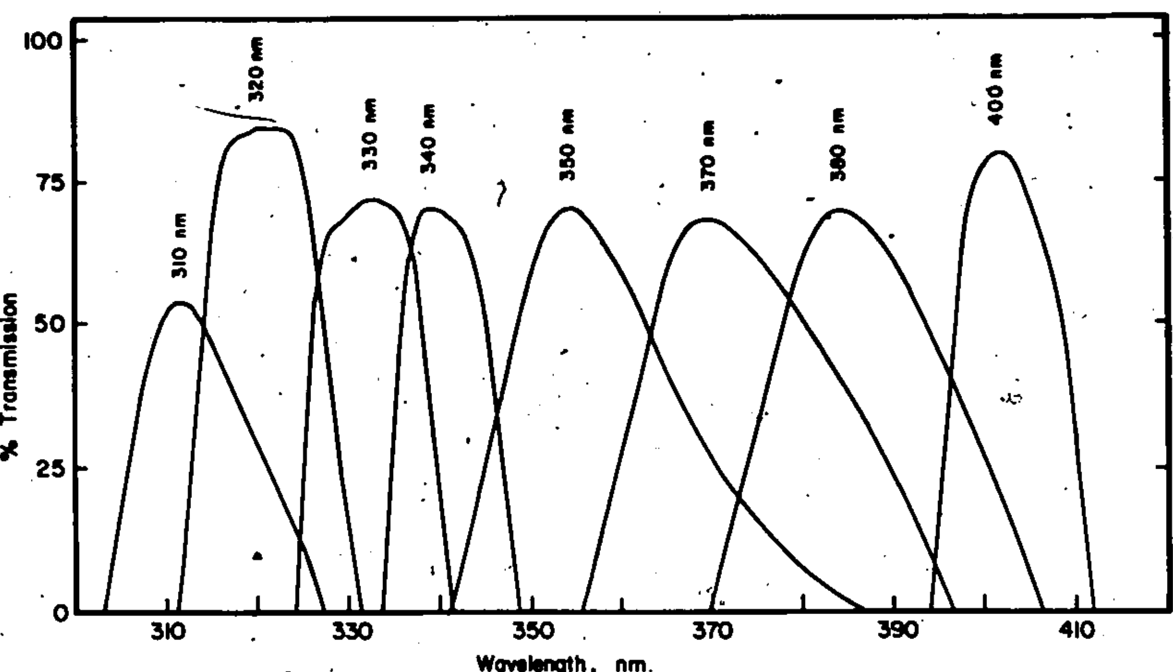
APPENDICES

Appendix A - Transmission spectra of Corion 10nm
half-bandpass interference filters.

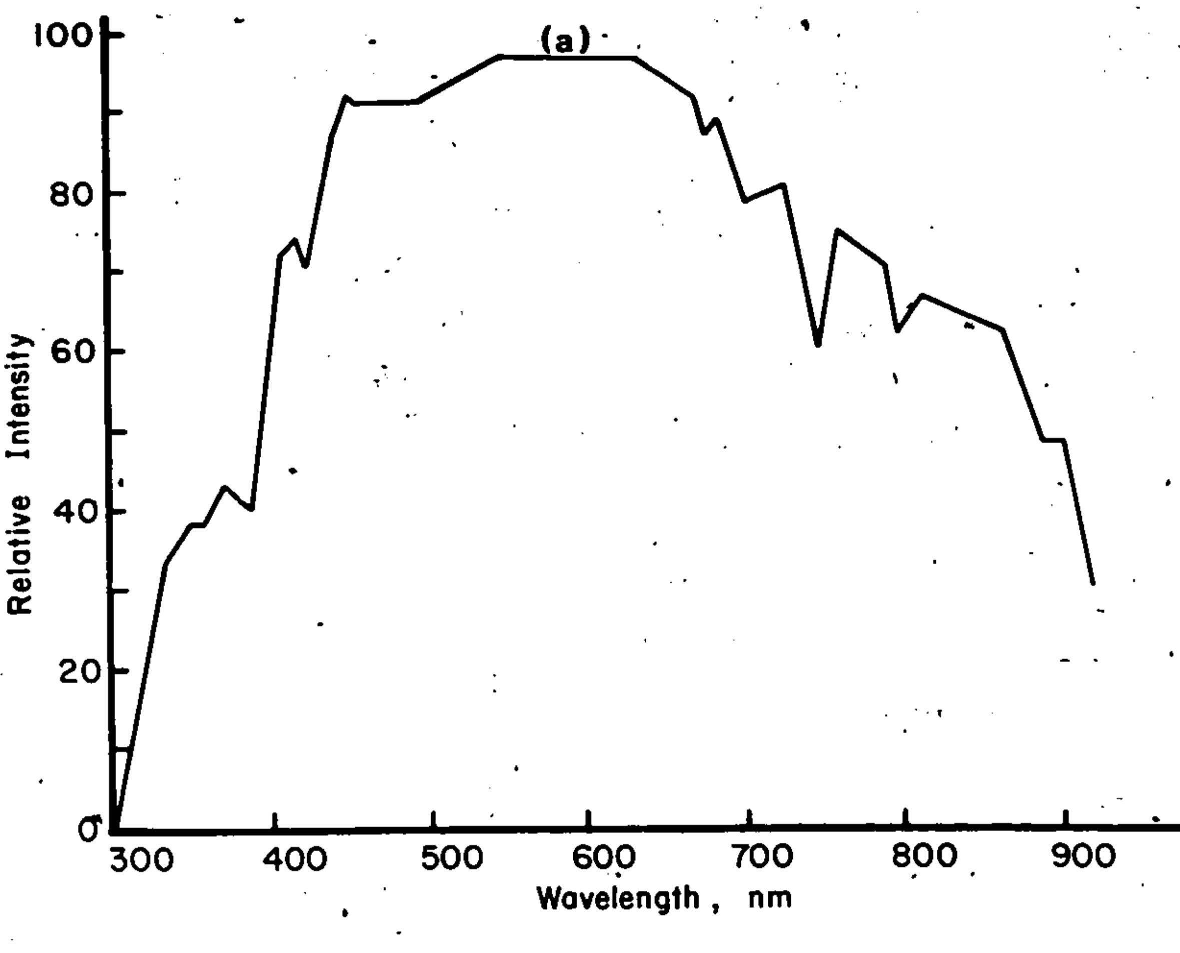
Figure 22. Transmisssion spectra of Corion Corp. interference filter series recorded in this study on a Pye-Unicam Sp8-100 dual beam spectrophotometer. The λ-value above each curve was the optimal transmission wavelength specified by Corion Corp. (Filters are suggested to have a half-band pass width of 10nm).



100
75
50
25
0
% Transmission
310 nm
320 nm
330 nm
340 nm
350 nm
370 nm
380 nm
400 nm
310
330
350
370
390
410
Wavelength, nm.

Appendix B - Solar and blacklight-blue fluorescent lamp emission spectra.

Figure 23. The curve (a) represents the spectral intensity of sunlight at the earth's surface. The lower scale correlates the ultraviolet, visible and near-infrared regions of the electromagnetic spectrum to wavelength (nm). (Adapted from diagrams by Jagger, 1977).


(a)
Relative Intensity
100
80
60
40
20
0
300
400
500
600
700
800
900
Wavelength, nm

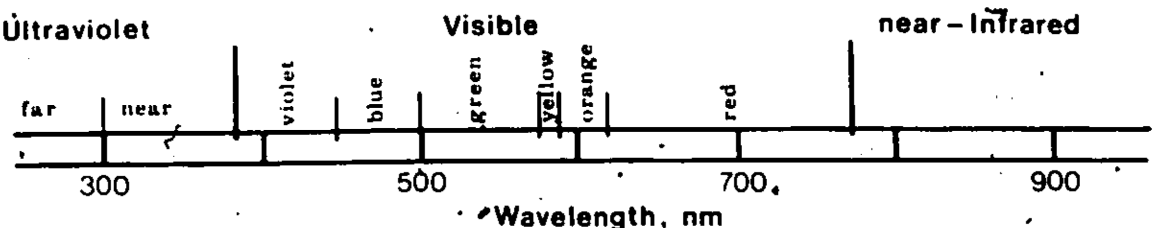
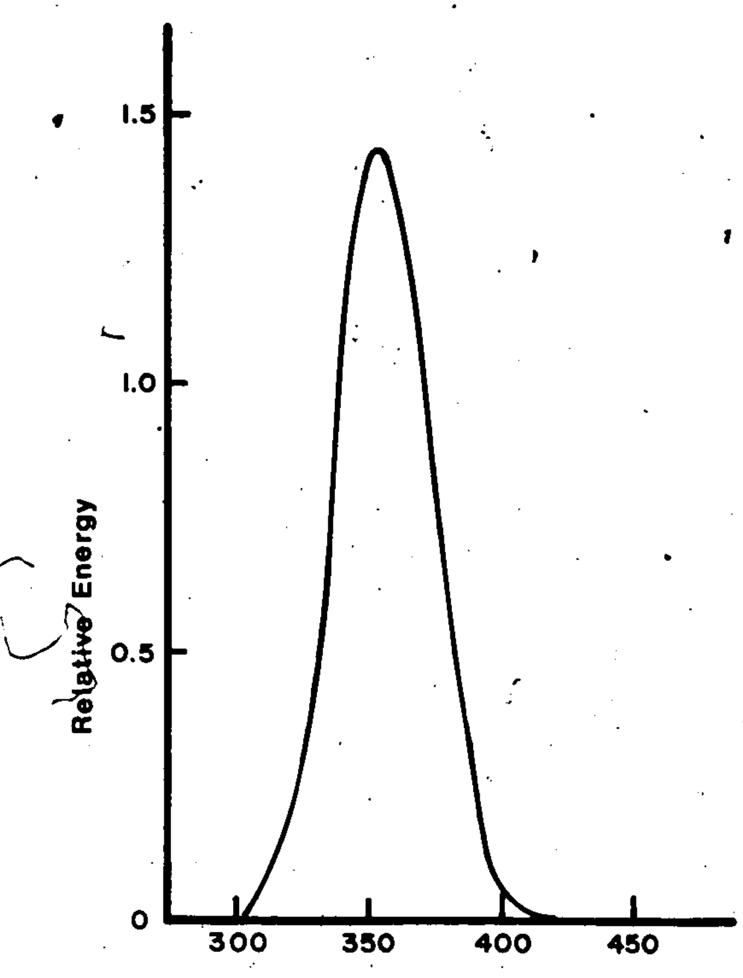
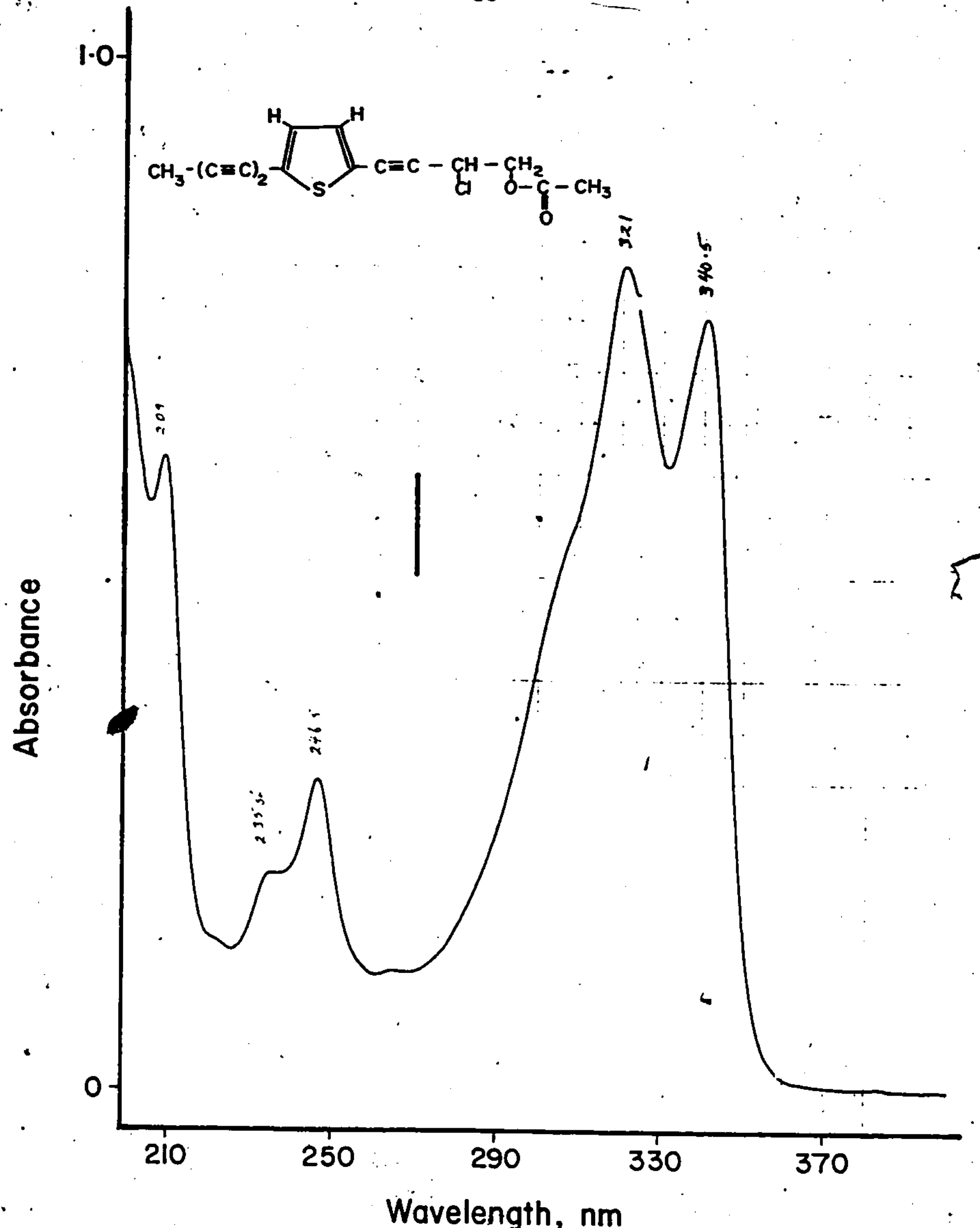

Ultraviolet
Visible
near-Infrared
far
near
violet
blue
green
yellow
orange
red
300
500
700
900
Wavelength, nm

Figure 24. Spectral output of 40-W T12 blacklight-blue lamps after 100 hrs of operation. (Courtesy of General Electric Co., reproduced from Jagger, 1977).


Relative Energy
1.5
1.0
0.5
0
300
350
400
450
Wavelength, nm

Appendix C - UV spectra of polyacetylene compounds from this study. (Heavy bars represent 0.1 ABS units. See Figure 4 for extinction coefficients at their UV-A λ_{max}.)



1·0
0
Absorbance
210
250
290
330
370
Wavelength, nm
321
340·5

1·0

343

$C \equiv C - CH = CH_2$

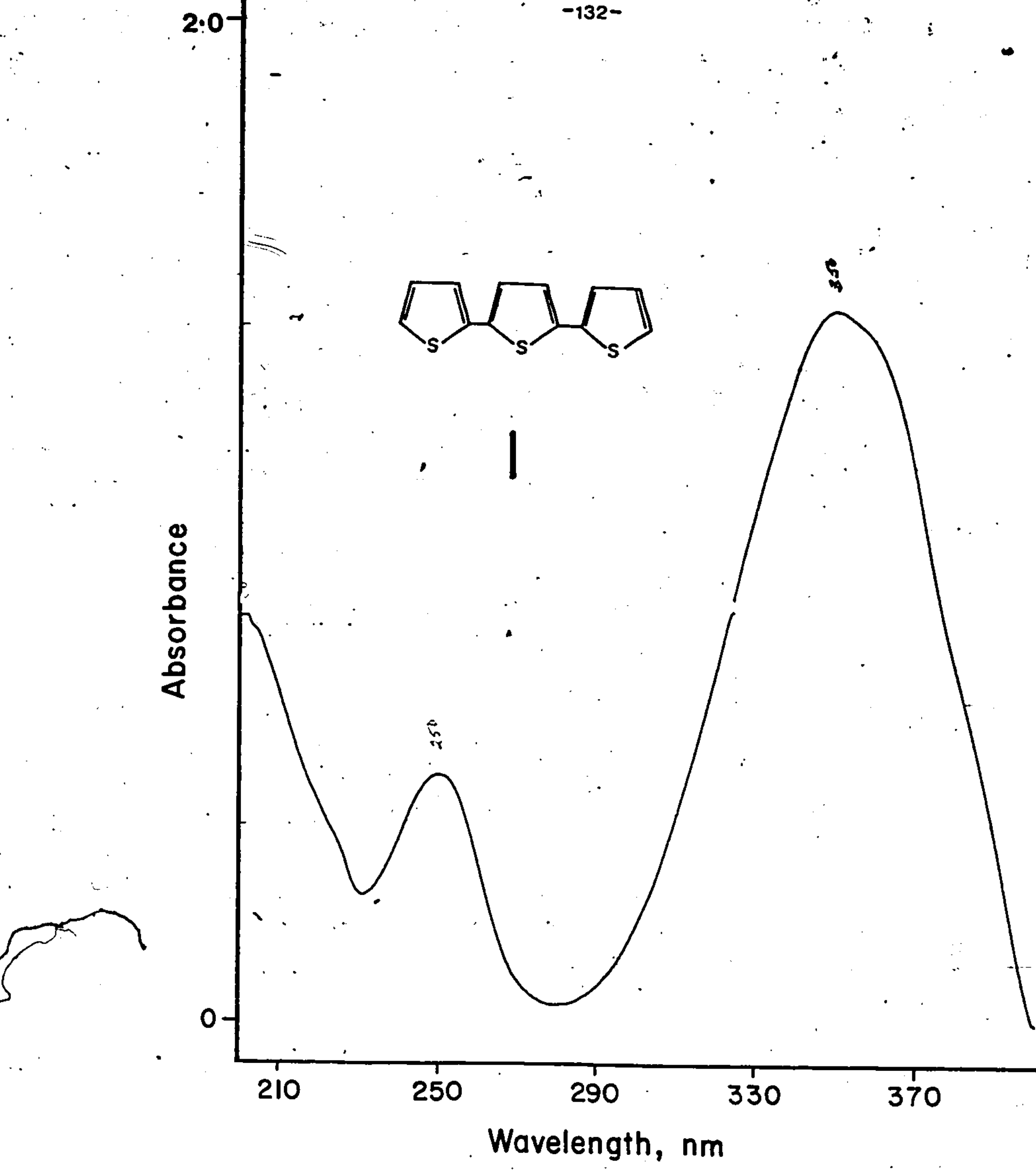
250

Absorbance

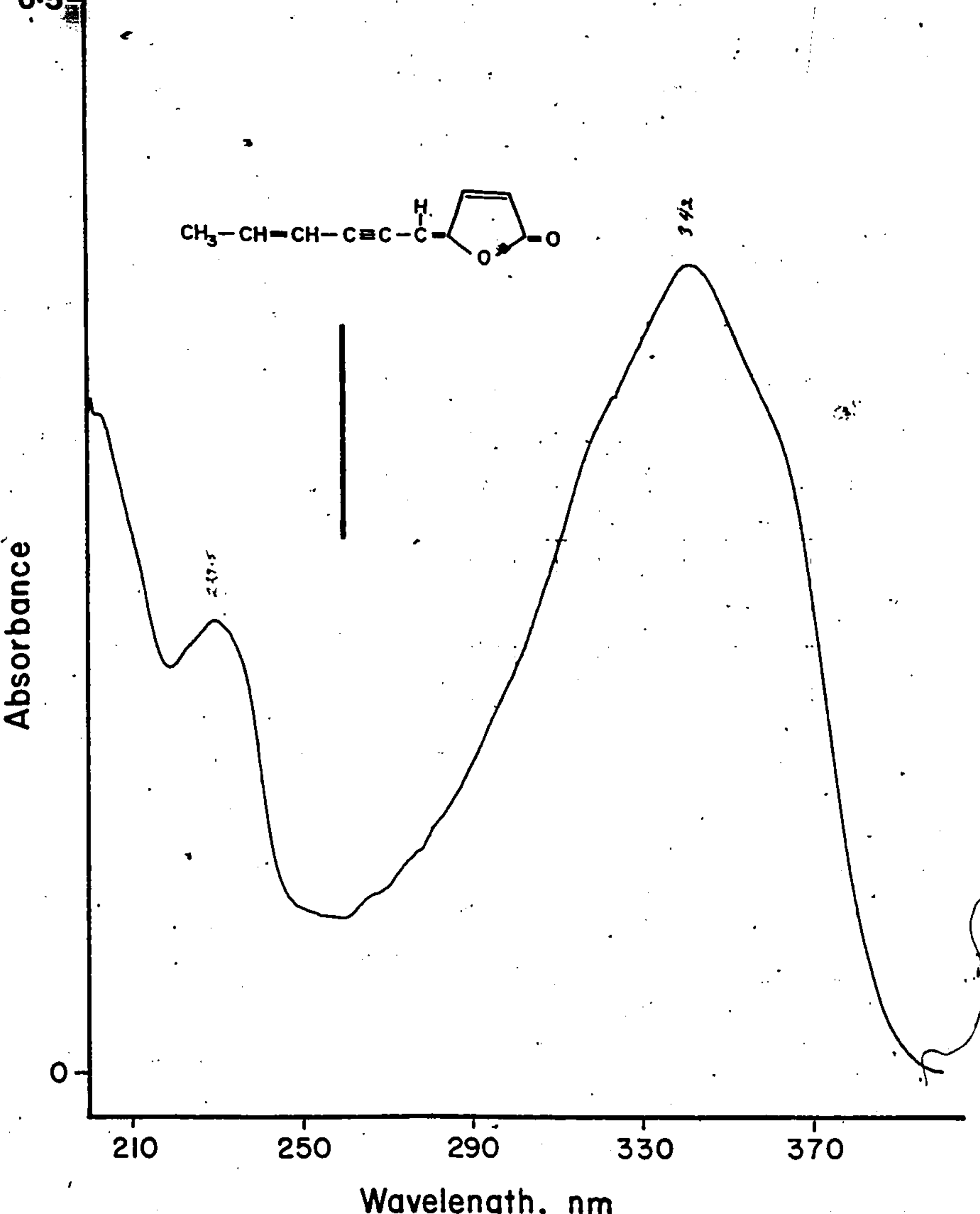
0

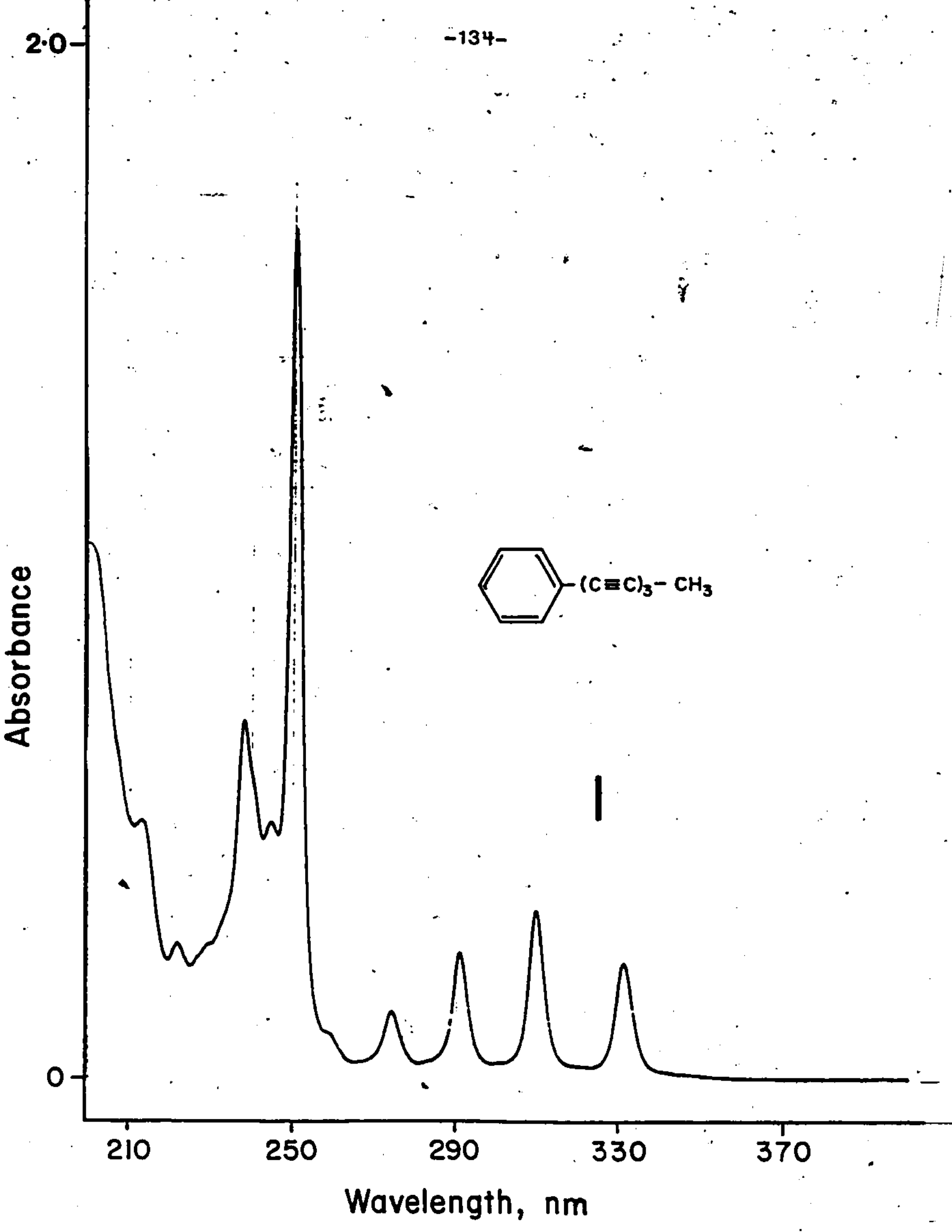
210 250 290 330 370

Wavelength, nm



2.0
0
Absorbance
S
S
S
I
250
350
210
250
290
330
370
Wavelength, nm


0·5
0
Absorbance
CH3—CH=CH—C≡C—C
H
O
O
342
227.5
210
250
290
330
370
Wavelength, nm


2·0
0
Absorbance
(C≡C)3– CH3
I
210
250
290
330
370
Wavelength, nm

1·0

Absorbance

0

205

230

239

252

269

274

297

317

$C_6H_5-(C\equiv C)_2-CH=CH-CH_2-O-C(=O)-CH_3$

210 250 290 330 370

Wavelength, nm

$C_6H_5-(C{\equiv}C)_2-CH{=}CH-CHO$

0.5

0

Absorbance

229.5 240 252 265 279 297 318

210 250 290 330 370

Wavelength, nm

$C_6H_5-(C\equiv C)_2-CH=CH-CH_2OH$

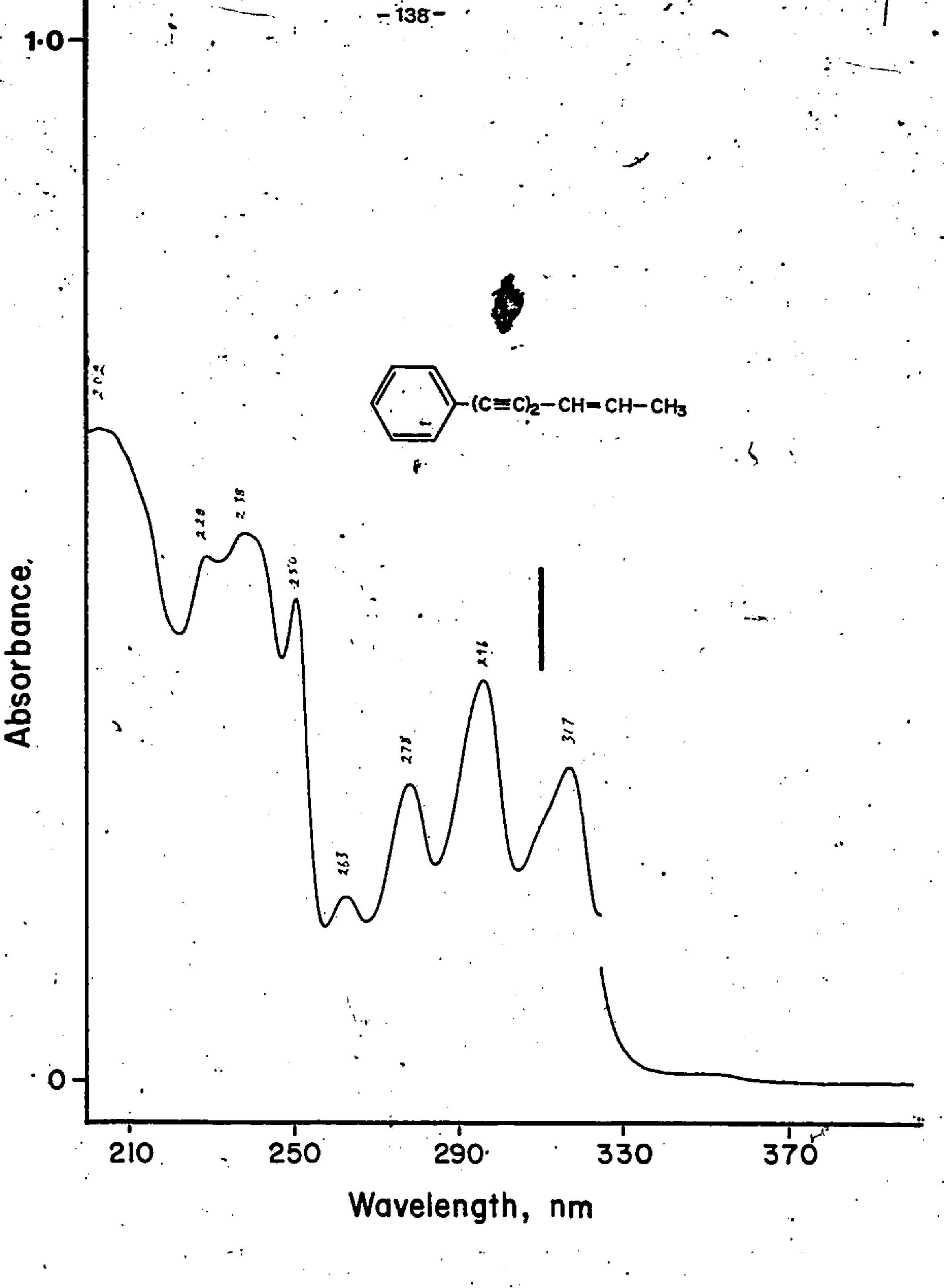
1·0

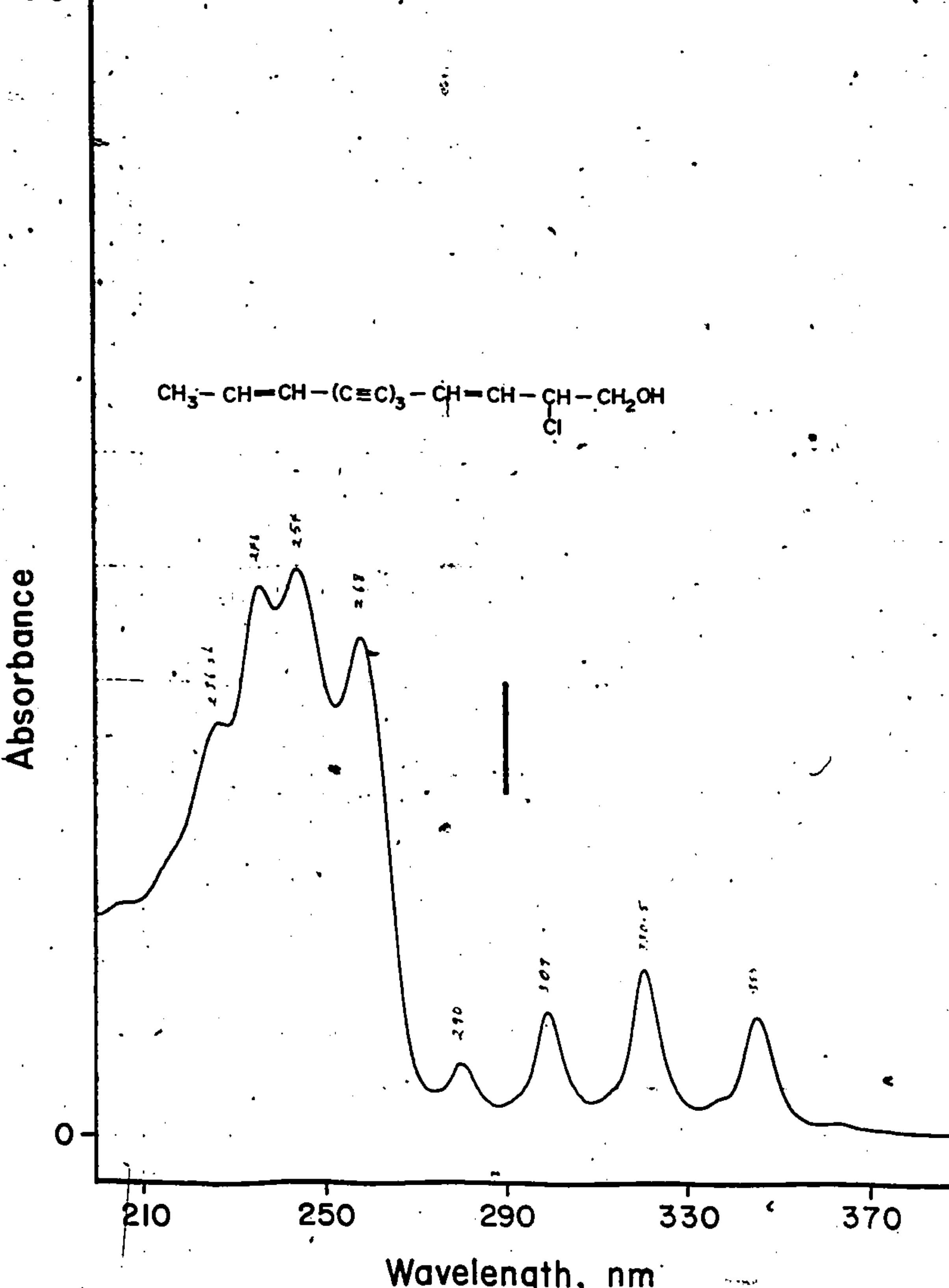
Absorbance

0

210 250 290 330 370

Wavelength, nm


1·0
0
Absorbance,
Wavelength, nm
210
250
290
330
370
(C≡C)2—CH=CH—CH3



1·0
0
Absorbance
$CH_3-CH=CH-(C\equiv C)_3-CH=CH-CH(Cl)-CH_2OH$
290
307
210
250
290
330
370
Wavelength, nm

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

255

244

285

303

323

346

2.0

Absorbance

0

210 250 290 330 370

Wavelength, nm

$CH_3CH{=}CH\,(C{\equiv}C)_2{-}CH{=}CH{-}C({=}O){-}O{-}CH_3$

1·0

Absorbance

0

209

223

244

256.5

291 sh

308

333 sh

210

250

290

330

370

Wavelength, nm

Appendix D - Chemical data on phenylheptatriyne purity from isolation proceedures in this study.

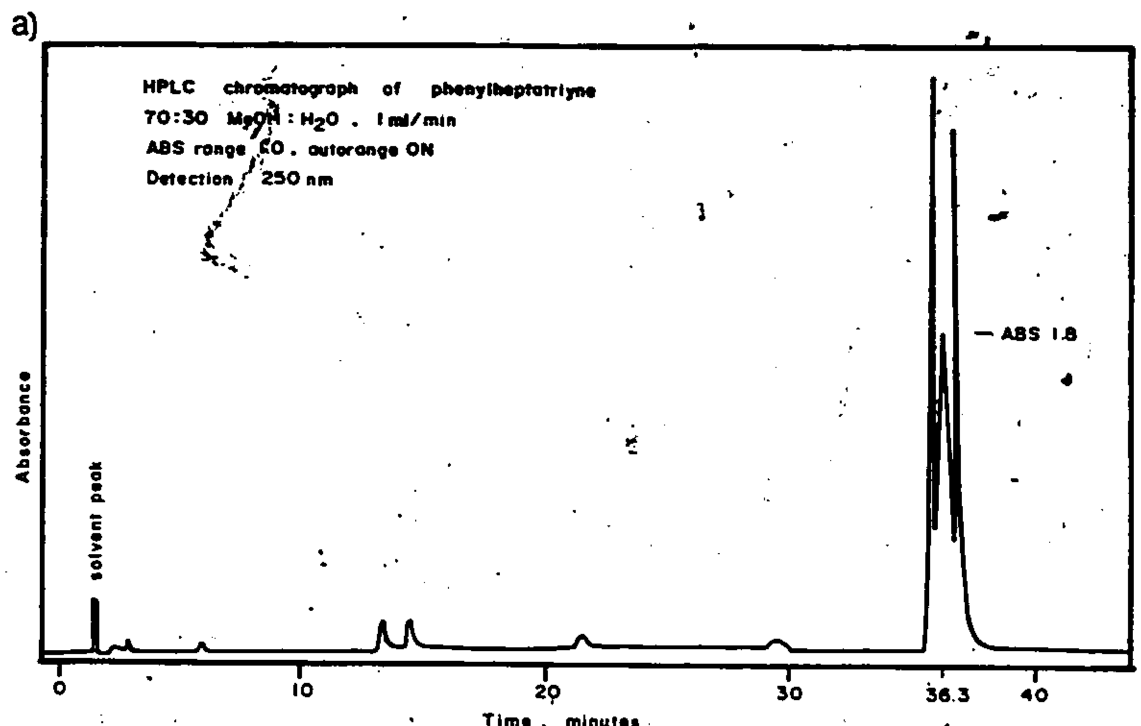
a) High performance liquid chromatograph of PHT with 70:30 MeOH & H_2O.

b) Mass spectrum of PHT crystals.

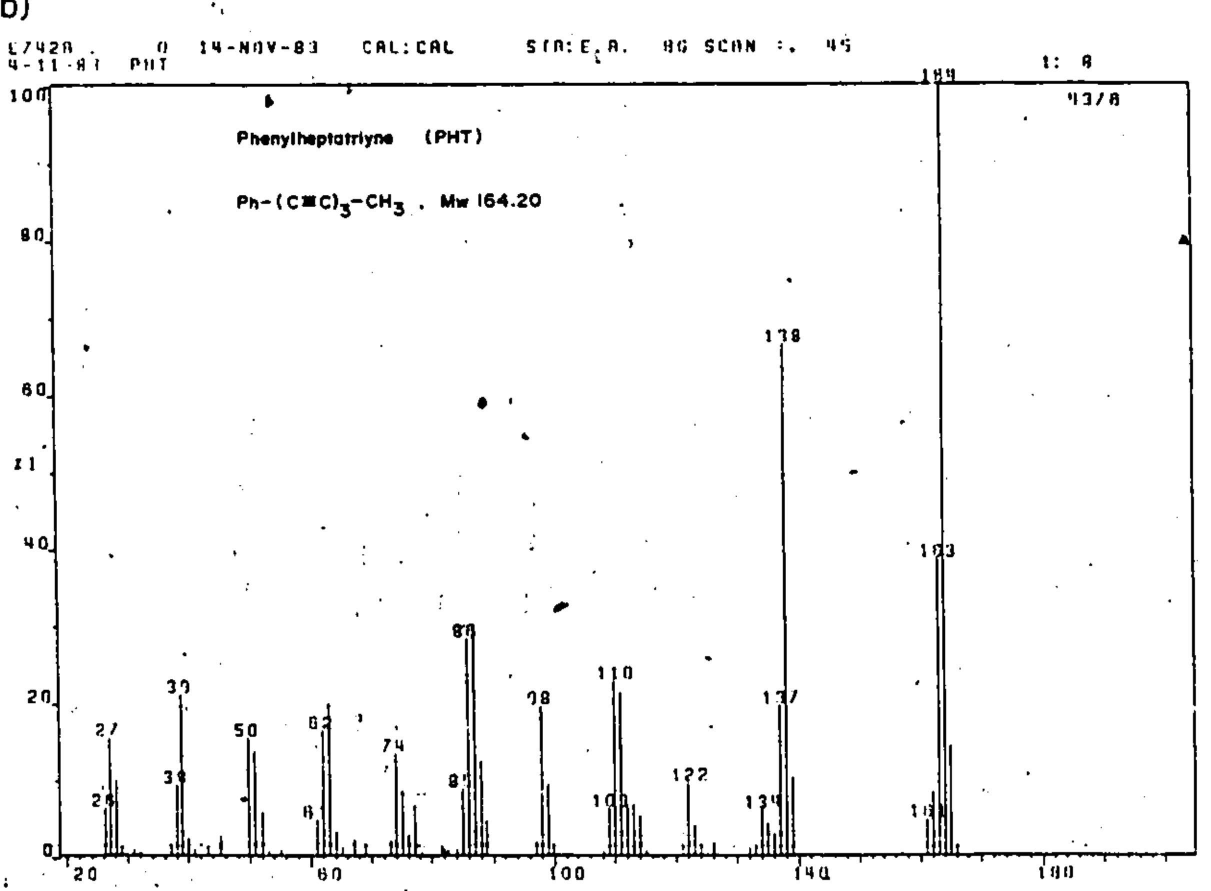
c) UV spectrum of PHT isolate.

d) Melting point determination.

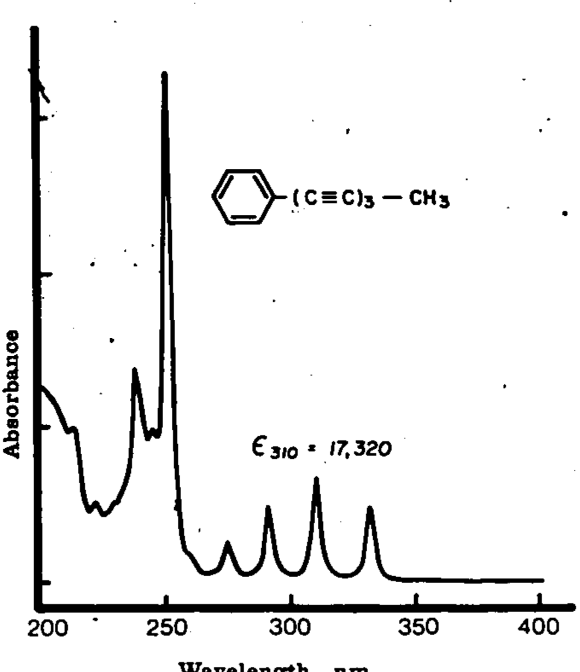
a)



b)



C)



D) The melting point determination yielded a melting point of 53°C, which is in agreement with Sorensen & Sorensen, 1958.