Photobiological Activity and Frameshift Mutagenesis of Umbelliferone, 7-hydroxycoumarin, with Bacterial and Mammalian cells.

by

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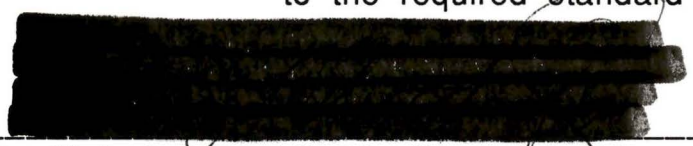
A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

We accept this thesis as conforming to the required standard



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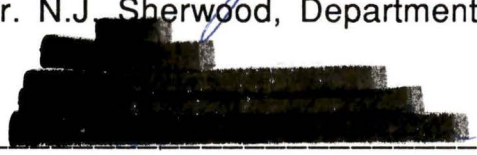

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ABSTRACT

Umbelliferone, 7-hydroxycoumarin, is a compound in the well known family of coumarins. It is widely distributed in nature and has had conflicting reports of photobiological activity, but has never been fully investigated. This study examined umbelliferone for photoactivity against various bacterial and mammalian test systems. Umbelliferone was found to be a photosensitizing agent at approximately 0.1 μg against an overlay of *E. coli B/s-1*, a DNA-repair deficient bacterium, but not active against wild type *E. coli B/r WP2 try-* until the compound was increased to 20 μg. A dose response curve with wild type *E. coli* was attempted but cell survival did not decrease significantly in a solution irradiation system. Survival did decrease to an average of 36% in an agar irradiation system over 2 experiments. No significant photosensitization could be found against Chinese hamster ovary cells even in 160 μg/ml of compound and after 20.1 kJ/m^2 of near ultraviolet light (NUV). Umbelliferone was an active frameshift mutagen at 60 μg/ml ($p < 0.05$) when tested against *E. coli lac-, z, thiamine* and exhibited a dose response relationship when increased sequentially to 175 μg/ml ($p<0.0006$) but was not active against *Salmonella typhimurium TA98* in the Ames test. To investigate the biological target of umbelliferone, the plasmid pTZ18R, encoding an ampicillin resistance gene, was irradiated with NUV in solution with umbelliferone and DMSO as a control. Umbelliferone at 15 μg/ml decreased the number of ampicillin resistant bacterial transformants by 95% versus the control after an NUV dose of 96.48 kJ/m^2. In conclusion, umbelliferone is a weak photosensitizing and frameshifting agent in certain strains of *E. coli*. As the two bacterial strains *E. coli B/s-1* and *B/r* exhibited a 200 fold difference in sensitivity to umbelliferone and as the ability of umbelliferone to react with pTZ18R DNA was clearly demonstrated, umbelliferone appears to be a weak DNA photoalkylating agent.

Examiners:

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

1D	one dimension
1E +04	1×10^4
1E +05	1×10^5
2D	2 dimensions
4-NQO	4-nitroquinoline 1-oxide
4-HAQO	4-hydroxy-aminoquinoline 1-oxide
8-MOP	8-methoxypsoralen
AVG	average
BHIB	brain heart infusion broth
BP	benzo(a)pyrene
CHO	Chinese hamster ovary fibroblasts designated as Puck's clone A. Cells are grown in McCoy's 5a medium from Terry Fox Laboratory
cont.	control
dH_2O	distilled water
DMBA	7, 12-dimethylbenz(a)anthracene
DMSO	dimethylsulfoxide
DMC	5, 7 dimethoxycoumarin
DNA	deoxyribonucleic acid
Expt.	experiment
EtOH	ethanol (all 95% in this study)
fig(s).	figure(s)
HPLC	high performance liquid chromatography
hr(s).	hour(s)
Irrad.	irradiated
MeOH	methanol
min(s).	minute(s)
millipore H_2O	water filtered through the Millipore MilliQ™ Water System CD0F01205
M	molar concentration
NA	nutrient agar obtained from Scott laboratory
NADP	nicotinamide adenine dinucleotide phosphate
nm	nanometer

NMR	nuclear magnetic resonance (both ^{1}H and ^{13}C NMR)
NUV	near ultraviolet light. All NUV light described in this study is from 2 parallel black light bulbs (GEC F20T12-BLB) emitting 13.4 Joules/m^2/sec. Wavelengths emitted are 310 - 380 nm, measured by chemical actinometry using ferri-oxalate with a Gaussian distribution round a peak at 350 nm
OD	optical density
ODS	octadecylsulfate
phosphate buffer	Sorrensen's phosphate buffer (0.07 M)
PUVA	topical application of psoralens and ultraviolet light therapy for psoriasis
Rt	retention time
S9 mix	a mix made from the supernatant fraction of a centrifuged Aroclor-induced rat liver homogenate combined with $MgCl_2$, KCl, glucose-6-phosphate, phosphate buffer, NADP, and NaH_2P0_4.
sec(s).	second(s)
S.D.	standard deviation
Std.	standard
TLC	thin layer chromatography on Merck silica plates without fluorescent indicator
TNA	tryptone nutrient agar
µg	microgram
µl	microlitre
µm	micrometre
Umb.	umbelliferone
UV	ultraviolet light
v/v	volume/volume
w/v	weight/volume
[ ]	concentration

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my husband, Vincent Low, who has been extremely patient, loving, and supportive and gave me a wide berth whenever I was in "one of those moods" from thesis writing. I would also like to thank my parents, Kwong Sing Ricky and Kim Tai Judy Lew, for their encouragement, love, and support throughout my academic career. They also gave our family an education more valuable than any obtained in an institution. I am also grateful to the rest of my family: Jacquelline Lew, Selina Jung, Cindy Lew, Robert Kung, and Park Jung whose love and humor helped keep me sane. I am also deeply indebted to my supervisor, Dr. M.J. Ashwood-Smith, for his guidance and financial support. My launch into the "academic" world would not have been possible without him. A huge thank-you goes to his research associates, Oluna Ceska and Pam Warrington. Their indispensable wisdom, guidance, and friendship will always be remembered. And finally, I would like to thank Dr. Will Hintz, Caihua Li, Dr. G.A. Poulton, and my committee members, Dr. N.M. Sherwood and Dr. P. Wan for their useful input.

DEDICATION

This thesis is dedicated to my loving and wonderful husband, Vincent Low and to my parents, Kwong Sing Ricky and Kim Tai Judy Lew.

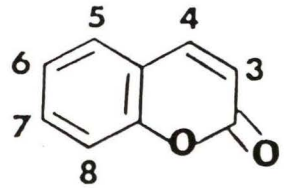
Introduction

Umbelliferone, 7-hydroxycoumarin, is a compound in the well known family of coumarins (see fig. 1). These plant produced compounds have functions which vary from regulation of seed/leaf germination, root growth, cellulose biosynthesis to acting as antimicrobial agents (Murray et al., 1982). Umbelliferone is found in approximately 34% of plants in the family Umbelliferae (Apiaceae) and 9% in the family Rutacea (Murray et al., 1982). Umbelliferone is a known antimicrobial agent and a building block in the synthesis pathway of the many more complex structured furanocoumarins such as psoralen and 8-methoxypsoralen depicted in fig. 2 (Feldman et al., 1973, Azakawa et al., 1960 and Suzuki, 1959 in Murray et al., 1982; Floss and Paikert, 1969 in Murray et al., 1982; Jurd et al., 1971).

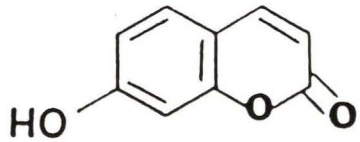
Topical application of psoralens and near ultraviolet light (320 - 360 nm), known as PUVA therapy, has been used as a highly effective treatment for many years for psoriasis. Although highly effective, this treatment has also been found to have possible oncogenic consequences (Epstein, 1979 in Ashwood-Smith et al., 1980; Stern et al., 1979 in Ashwood-Smith et al., 1980; Strauss et al., 1979). By alkylating DNA upon photoexcitation, cell proliferation of psoriatic epidermal cells is effectively blocked; however, unwanted mutagenic effects can also occur.

The antimicrobial activity of many coumarins and furanocoumarins has also been mostly attributed to their photobiological ability. These planar molecules intercalate in the dark between base pairs of double stranded deoxyribonucleic acid (DNA) and upon near-ultraviolet light radiation alkylate pyrimidines via formation of cyclobutane dimers (Morrison, 1990; Ivie, 1987; Ou et al., 1978). Furanocoumarins have been implicated as frameshift mutagens in the dark and as photomutagens, photoclastogens, and lethal photosensitizers of cells and bacteria. They have also been shown to cause contact photodermatitis in humans and herbivores (Ashwood-Smith et al., 1992; Zobel and Brown, 1990).

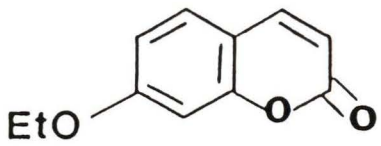
Figure 1. Chemical structures of some simple coumarins.



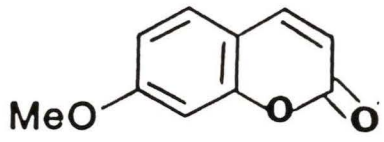
COUMARIN



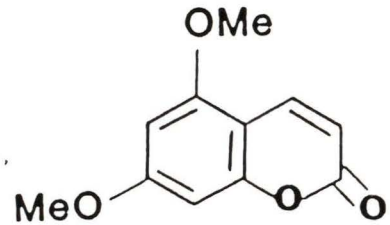
UMBELLIFERONE



7-ETHOXY COUMARIN

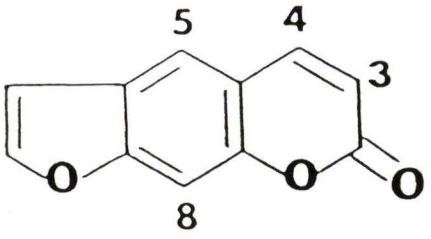


7-METHOXY COUMARIN

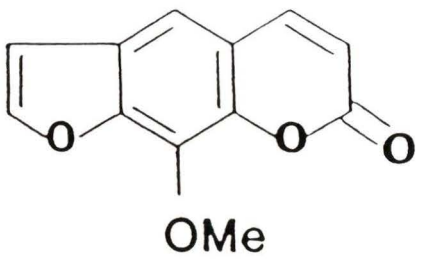


5,7-DIMETHOXY COUMARIN

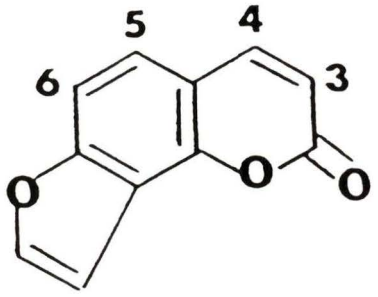
Figure 2. Chemical structures of some linear furocoumarins: psoralen and xanthotoxin and an angular furocoumarin: angelicin.
xanthotoxin= 8-methoxypsoralen.



PSORALEN



XANTHOTOXIN



ANGELICIN

Many instances of antimicrobial activity of umbelliferone have been documented but the mechanism of action has never been fully investigated. In our laboratory, the photosensitizing ability of this compound in bacterial and fungal systems has been noted on numerous occasions in previous research (Ceska et al., 1992) but has never been documented by any other researcher in the literature. The widespread occurrence of this compound in nature and the equally ubiquitous use in research and industry make it necessary to investigate the photobiological activity of this compound.

A review of the literature concerning umbelliferone's occurrence, chemical characteristics, known antimicrobial activity, and other biological activity is discussed first. A brief overview of its uses in industry and research is then discussed to highlight the importance of this compound.

Occurrence in Nature

Umbelliferone occurs extensively in nature. As well as being found in the families Umbelliferae and Rutaceae, it is also found in other families such as Asteraceae, Mimosaceae, Caesalpiniaceae, Fabaceae, Caryophyllaceae, Euphorbiaceae, Hippocastanaceae, Oleaceae, and Thymelaeaceae but to a lesser degree (Murray et al., 1982). Biosynthesis of umbelliferone and other simple coumarins can originate via shikimic and chorismic acids, phenlyalanine or cinnnamic acids. Phenlyalanine is converted via phenlyalanine ammonia-lyase to trans-cinnamic acid. Trans-2-2'-hydroxycinnamic acid and trans-2-4'-hydroxycinnamic acid and their glycosylated forms have been shown to be precursors to umbelliferone via various hydroxylation reactions (Brown, 1962 in Murray et al., 1982; Murray et al., 1982; Austin and Brown, 1973). Umbelliferone therefore also occurs in plants in a bound form: 7-*B*-D-glycosyloxycoumarin (Austin and Meyers, 1965 in Murray et al., 1982).

Chemical Characteristics

Umbelliferone has many chemical characteristics which make it very useful in both research and industry. Umbelliferone has an amphipathic structure which makes it soluble in polar and relatively nonpolar solvents. Umbelliferone has strong absorption peaks at 217 and 325 nm with weak peaks at 240 and 255 nm and an absorption spectrum is enclosed in the appendix (Murray et al., 1982; Sen and Bachi, 1958). When UV spectra of umbelliferone are recorded in alkaline medium, the absorption maxima shows a marked bathochromic shift from 325 to 372 nm which occurs upon electron delocalization of the phenoxide ion (see fig. 3). This UV shift in absorption in different pH's makes umbelliferone applicable in many research/industry uses.

Umbelliferone's intense blue fluorescence visible under UV light is due to the excited singlet state of the phenolate form of the compound (see fig. 3) (Beddard et al., 1977 in Li et al., 1981). Under UV, the neutral form does not fluoresce. This again conduces applications to numerous research/industrial uses. Both neutral and charged forms are pale yellow in visible light (Dean, 1957).

Biological Activity

Furanocoumarins, the most notorious phototoxins, are thought to have arisen as defenses against plant pathogens and herbivores (Berenbaum and Feeny, 1981; Berenbaum, 1981; Berenbaum, 1978). Both the furan and pyrone ring double bond (see fig. 2) are potential alkylating agents: the linear furanocoumarins are able to form both mono- and diadducts (crosslinks) whereas the angular furanocoumarins form monoadducts only (for diagrams and details, see Morrison, 1990; Murray et al., 1982; Ou et al., 1978; Ashwood-Smith et al., 1977). Some simple coumarins have been shown to have photobiological activity: 5, 7 dimethoxycoumarin (DMC) and 7-methoxycoumarin (see fig.1). Coumarins have only one reactive bond and therefore can only form monoadducts as shown by fig. 4. DMC

Figure 3. Resonance structures of umbelliferone (7-hydroxycoumarin) in alkaline conditions.

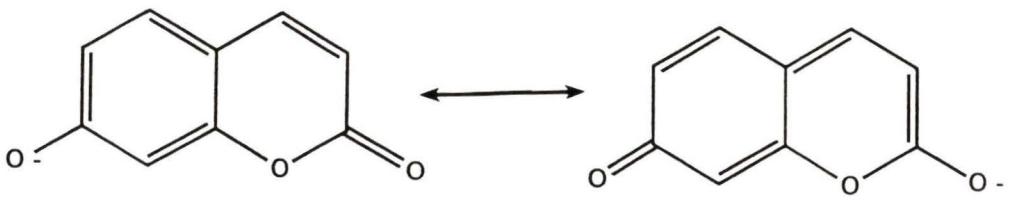
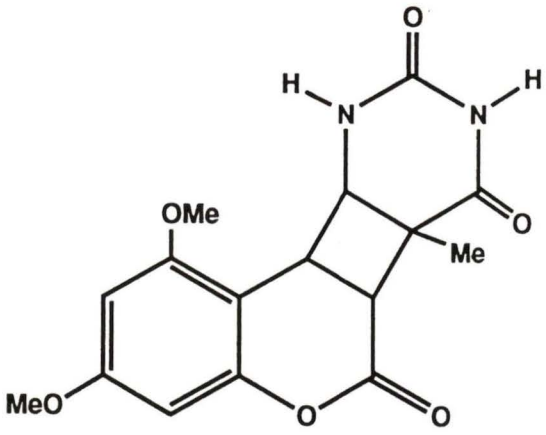

O -
O
O
O
O
O -

Figure 4. Chemical structure of the 3-4 cycloaddition product of the lactone ring of 5,7-dimethoxycoumarin with thymine.


O
H
N
N
H
O
OMe
Me
MeO
O
O

differs from umbelliferone in having a methoxy group at the 7 position and an additional methoxy group at the 5 position. Because of the similarity in structure, DMC's documented biological activity is discussed in more detail.

Evidence for DMC binding to DNA is extensive. The mechanism, as with furanocoumarins, entails the coumarin and DNA forming an intercalated complex (Ou et al., 1978). This does not require light and is thus called a dark reaction which can cause frame-shift mutations (Ashwood-Smith et al., 1983; Ashwood-Smith, 1978; Bridges and Mottershead, 1977). The geometry of this intercalated complex has been well detailed by many authors using molecular mechanics. (For details and diagrams, see Cadet and Vigny, 1990; Demaret et al., 1989). Light activates the 3-4 lactone bond to form a monoadduct with the C5-6 bond of a pyrimidine as shown by fig. 4. Preferential cycloaddition sites for DMC and furanocoumarins are pyrimidines with preferential intercalation sequences being alternate purine and pyrimidine: d(A-T) or (G-C) sequences (Sage and Trabalzini, 1991; Murray et al., 1982; Ou et al.,1978). Ou et al., (1978) found that DMC reacts with DNA via its singlet excited state.

Furanocoumarins and DMC cycloadditions give transcription-terminating lesions (Nocentini, 1978 in Murray et al., 1982) and block movement of DNA polymerase along the modified DNA template (Song et al., 1979 in Murray et al., 1982; Ou et al.,1978) Furanocoumarins and DMC have also been shown to cause sister chromatid exchanges and lethal photosensitization in Chinese hamster ovary cells. (Ashwood-Smith et al., 1983; Ou et al., 1978; Harter et al., 1976). Harter et al., (1976) found that DMC is a highly active photosensitizer in *Bacillus subtilis*.

Umbelliferone's capability of C3-4 cycloadditon in vitro has also been investigated with naked DNA and RNA and umbelliferone has been shown to be a weak photoadditive agent. However, its full biological activity has never been investigated (Marciani et al., 1971). Umbelliferone was reported to have no effect in increasing

sister chromatid exchanges in Chinese hamster ovary cells in a paper published by Sasaki et al. (1987) but light activation was not used.

Furanocoumarins and DMC are nonspecies-specific photoalkylating agents of DNA. In single celled organisms, such interactions may lead to cellular death, inhibition of cellular division, and mutations. In multicellular organisms, the photosensitizing effects are thought to be limited to dermal and subdermal tissues. In mammals and in man, toxicological effects can vary from erythema, skin blistering, to carcinogenicity (World Health Organization, 1987; Ashwood-Smith et al., 1979 and 1980). The World Health Organization has classified 8-MOP as a Group 1 carcinogenic agent in humans: i.e. an agent that produces genetic or related effects in exposed humans indicative of DNA or chromosomal damage (1987).

Another simple coumarin found to be photobiologically active is 7-methoxycoumarin (see fig.1). This compound produced phototoxic reaction and photocontact sensitization in humans at 5 µl of a 5% (w/v) solution and 2 Joules/cm^2 (Kaidbey and Kligman, 1981). Ceska et al., (1992) also found that 7-methoxycoumarin was a very reactive photosensitizing agent in many bacterial and fungal systems. Because of the photobiological activity of these simple coumarins, the possibility for significant photobiological activity for umbelliferone exists.

Umbelliferone's documented biological effects have been contradictory and varied. Many experiments studying its biological activity have not specified light conditions during the assay. As stated previously, Marciani et al., (1971) reported significant cycloaddition with DNA and RNA in test tubes but its biological significance was never investigated. Table 1 summarizes known biological activity of umbelliferone against microorganisms/fungi.

As shown in table 1, umbelliferone has been reported both as testing positive and negative in antimicrobial/antifungal assays

Table 1: Biological Activity of umbelliferone against various organisms as found by different investigators

Organism	Investigator*									
	1	2	3	4	5	6	7	8	9	10
Aerobacter aerogenes							-			
Bacillus megatherium	+						-			
Bacillus subtilis	+				+					
Escherichia coli B/r	+				+		-			
Escherichia coli B/s	+									
Klebsiella pneumonia			+							
Micrococcus luteus, M. lysodeictus							-			
Micrococcus phlei	-									
Salmonella typhimurium TA100			-							
Serratia marcescens							-			
Staphylococcus aureus	-						-			
Staphylococcus marcescens	-				-					
Alternaria sp.									-	
Aspergillus flavus, A. glaucus						-				
Aspergillus niger	-					-			-	
Aspergillus sydowii	+									
Botrytis cinerea						-				
Brucella sp.								+		+
Byssochlamys fulva									+	
Candida albicans	-			+						
Candida chalmseri, C. tropicalis						-				
Curvularia lunata									-	
Hanseniaspora melligeri						-				
Hansenula anomala						-				
Penicillium chrysogenum						-				
Penicillium claviforme	+									
Penicillium notatum	+									
Pichia chodati						-				
Rhizopus senti						-				
Saccharomyces cerevisiae	+					-				
Saccharomyces mellis, S. rosei						-				
Sclerotinia sclerotiorum	-									
Torula utilis						-				
Zygosaccharomyces barkeri						-				
Zygosaccharomyces japonicus						-				
Murine L1210 cells		-								

biological activity +
no biological activity -
* key for investigators on proceeding page

Table 2. Key to Investigators of umbelliferone's biological activity as described by Table 1*

1	Ceska et al., 1992
2	Kang and Ahn, 1986
3	Voogd et al., 1980
4	Szalontai et al., 1977
5	Fischer et al., 1976
6	Jurd et al., 1971
7	Dadak and Hodak, 1966
8	Duquenois et al., 1965
9	Chakraborty et al., 1961
10	Greib and Hazard, 1954

*Full references given in bibliography

against *Candida albicans*, *Escherichia coli*, *Bacillus megatherium* and *Saccharomyces cerevisiae*. Umbelliferone was a reported antimicrobial agent against *Brucella sp.* and *Bacillus subtilis* (Fischer et al., 1976) but has also been reported as ineffective on the following gram-positive microorganisms: *Staphylococcus aureus*, *Micrococcus luteus*, *M. lysodeicticus*, *Bacillus megatherium* and the following gram-negative bacteria: *Aerobacter aerogenes* and *Serratia marcescens*.

Voogd et al., (1980) tested the mutagenic action of umbelliferone against *Klebsiella pneumoniae* and the Ames plate incorporation test using *Salmonella typhimurium* TA100 (both base substitution mutation tests). Umbelliferone tested positive at a concentration of 0.8 g/l on *Klebsiella* but negative against *Salmonella*. They suggested that the discrepancy between results may have been due to *Salmonella* growth inhibition by umbelliferone.

Umbelliferone was also found ineffective against growth inhibition of numerous fungi: *Zygosaccharomyces sp*, *Candida sp.*, *Saccharomyces sp.*, *Aspergillus sp.*, *Curvularia lunata* and many others but active in *Byssochlamys fulva*. Kang and Ahn (1986) also found that umbelliferone was not cytotoxic on murine L1210 cells and theorized that because of the presence of the hydroxy group and its position, umbelliferone was not active. As stated previously, light conditions were not mentioned in all of the above biological studies which may account for the conflicting data.

On the contrary, Ceska et al., (1992) in our laboratory found significant antimicrobial/antifungal activity with NUV activation. Umbelliferone was found slightly active against *Bacillus subtilis* and *B. megatherium*, *Penicillium claviforme* mycelium and *Aspergillus sydowii* mycelium, fairly active against *E. coli B/r* and *E. coli* B/s_{-1} and just discernible for *Penicillium notatum* mycelium and *P. claviforme* spores. It was negative for *Staphylococcus aureus*, *S. marcescens*, *Micrococcus phlei*, *Candida albicans*, *Penicillium notatum* spores, *Aspergillus niger* spores and mycelium,

A. sydowii spores and *Sclerotinia scelorotiorum.* All dark controls were negative.

Because of umbelliferone's widespread occurrence in plants, its contradictory documentation of antimicrobial/antifungal activity and our preliminary findings of its photosensitization ability, it was considered necessary to investigate its role as a photomutagen. A brief overview of umbelliferone's other biological effects and its importance in research and industry is, however, discussed next to highlight the importance of obtaining more information about this molecule.

Other documented biological effects

Umbelliferone is believed by some investigators to function both by immunologic and non-immunologic mechanisms in reducing tumor growth (Marshall, 1992; Sharifi et al., 1992 in Marshall, 1992). In humans, umbelliferone is being used in clinical trials in Ireland to combat various forms of cancer. Patients are given oral doses ranging from 100 to 3500 mg per day. Claims have also been made for cytostatic effects on various cell lines (Marshall et al., 1992 in Marshall, 1992; Moran et al., 1992 in Marshall, 1992) but these reports have been conflicting (Sasaki et al., 1987; Gerson et al., 1986). It is postulated that umbelliferone may mediate macrophage function via cell receptors or interfere with cell division machinery but evidence is scanty, thus far, and the mechanism of action is purely speculative (Marshall, 1992; Sasaki et al., 1987; Gerson et al., 1986).

Umbelliferone has also been reported to have inhibitory effects on DMBA induced mammary tumors in rats and benzo(a)pyrene induced neoplasia of the forestomach in mice. However, again, the process of inhibition is unknown (Feuer et al., 1976 in Nair et al., 1991; Wattenberg et al., 1979). Possibilities include modification of mixed function oxidase enzymes which turn compounds like benzo(a)pyrene into strong nucleophiles. Degradative metabolism in

mammals, one major aspect of umbelliferone research, will later be briefly discussed.

Anti-mutagenic effects of coumarin and umbelliferone on mutagenesis induced by 4-NQO or UV irradiation in *E. coli* has been reported by Ohta (1983). All procedures were done in the dark following UV irradiation with 4-NQO. An 83% decrease in the number of 4-NQO-induced revertants was observed with umbelliferone plated at 10 μg/ml without any decrease in cellular viability. 4-NQO is metabolically activated to 4-HAQO by bacterial nitroreductase and thus umbelliferone may interact with this enzyme to reduce 4-NQO conversion (Ohta et al., 1983). Thus, because of umbelliferone's increasing importance in antitumorigenicity, any biological mechanisms by which it acts must be investigated.

Extensive Uses in Research/Industry

Umbelliferone is also important for its wide usage in research and industry. Umbelliferone is used in dye lasers, as a fluorescent dye in many cosmetics and as an optical whitener (Budavari, 1989; Slawinska and Slawinski, 1989). Umbelliferone and/or its conjugates/derivatives are also used in numerous other research applications. For example, umbelliferone is used in human research to determine serum levels of drugs such as theophylline, amikacin, gentamicin, and kanamycin (Place et al., 1984; Li et al., 1981; Thompson and Burd, 1980; Voogd et al., 1980; DeCastro et al., 1968). Umbelliferone when conjugated with the drug is nonfluorescent and can be bound by antiserum to prevent hydrolysis by *B*-galactosidase. Unbound substrate can be hydrolyzed by the enzyme which releases a fluorescent umbelliferone product that is directly proportional to the nonconjugated drug concentration.

This principle involving the reaction of a conjugated non-fluorescent form of umbelliferone with an enzyme in a target area

to yield a fluorescent product has also lent itself to research in areas such as influenza C virus O-acetylesterase activity and Na, K-dependent adenosine triphosphate phosphohydrolase activity (Garcia-Sastre et al., 1991; Boldrey et al., 1984; Kohen et al., 1984). Umbelliferone is also used to determine lysosomal hydrolase activity in serum/synovial fluid of rheumatoid arthritis patients (Peicheva et al., 1986).

Umbelliferone is used extensively as an intracellular brain pH probe in research animals to analyze the pathway of fat soluble substances across the blood-brain barrier and to determine lactate and pH in the brain and other tissues (Paschen et al., 1987). Umbelliferone's pKa is 7.5 and the absorbance maxima and fluorescence spectra of the neutral and anion form differ (Paschen et al., 1987). Thus the pH of the indicator's environment along its pathway into and out of brain tissue can be determined from comparative analyses of fluorescence upon 340 and 370 nm excitation at different points due to different fluorescence of the protonated and non-protonated form (Sundt and Anderson, 1980).

As stated previously, umbelliferone has also been important in the research of degradative metabolism and detoxification (Sharp et al., 1984; Ohta et al., 1983; Shilling et al., 1969). Cytochrome P-450 and other mixed function oxidase enzymes participate in coumarin and other ring structure metabolism via formation of epoxides. These intermediates then undergo hydroxylations to increase polarity of these compounds for excretion. Many of these pathways metabolize relatively nonreactive compounds into very reactive and biologically damaging species. Hydroxylated compounds can be excreted by themselves or as ethereal sulphates or glucuronides. In man, 7-hydroxycoumarin is glucuronidated for excretion (Ritschel and Hoffman in Marshall, 1992; Murray et al., 1982). Umbelliferone is used to determine liver functions such as transferases and hydrolases as well as those associated with glucuronide, sulfate and glutathione conjugation (el Mouelhi and Kauffman, 1986). Some studies have shown that umbelliferone may be an antimutagen in

preventing the formation of reactive forms of 2-aminoanthracene and 4NQO by possible interference with liver microsome enzymes (Wall et al., 1990; Ohta et al., 1983).

Overview of thesis research

This research entails investigating the biological activity of umbelliferone in the following assays:

1) lethal photosensitization of *E. coli B/s-1*
2) lethal photosensitization of *E. coli B/r*
3) frameshift mutagenesis of *E. coli lac*$^-$
4) frameshift mutatagenesis of *Salmonella typhimurium* TA98 in the Ames test
5) lethal photosensitization of CHO cells
6) inactivation of the ampicillin resistance gene of the plasmid, pTZ18R

A brief introduction to each biological assay is given prior to a description of the technique. Umbelliferone was first purified and its character confirmed prior to testing. Because this compound was found to form breakdown products upon NUV irradiation, irradiated umbelliferone was also tested on TLC and HPLC and for its absorbance/fluorescence spectra for characterization as well as in some biological assays for photoactivity.

Materials and Methods

Characterization

Umbelliferone was obtained from Fluka Chemie AG in Switzerland and checked for purity by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). 1 mg/ml standards in HPLC grade methanol were made and spotted in various amounts from 1 to 100 μg on Merck K 60 silica plates with fluorescent indicator when checking for purity and without fluorescent indicator when plate used for biological assays. All standards were made fresh before use and stored in the dark at 4°C. All procedures were performed under red light when required.

TLC plates were developed in various solvents and the following system was found to have the best resolution: 2 dimensional analysis with first I) chloroform/ethyl acetate 5:1 followed by II) hexane/acetone 1:1. Some samples were developed in hexane/acetone 1:1 in both directions depending on desired separation of compounds. All solvents were HPLC grade and obtained from Fischer or BDH. All compounds in TLC were visualized with UV light at 254 nm. TLC's of irradiated umbelliferone later described were also developed in the same manner with above Merck plates.

Umbelliferone was also analyzed on reverse phase HPLC. A Beckman 126 instrument with an ultrasphere ODS 5 μm pore analytical 4.6 mm x 25 cm column or semi-preparative column (10 mm x 25 cm) was used. The analytical column was used to test umbelliferone for purity while both the analytical and semi-preparative columns were used to test irradiated umbelliferone samples described later. Because of the possible trace amounts of photodegradation products being formed, it was thought a more concentrated injection of the irradiated sample in a semi-preparative column would allow better resolution and ultimate detection.

Flow rates used were 1 ml/min. for the analytical column and 3 ml/min. with the semi-preparative column. A Beckman 166 UV detector was used to detect compounds at 240, 254, and 325 nm. Standards were dissolved in the mobile phase at 10 µg/ml for analytical runs and 20 µg/ml for semi-preparative runs. Samples were tested in various isocratic mobile phases from 95% CH_3CN: 5% H_2O to 40% CH_3CN: 60% H_2O and 70% methanol: 30% H_2O to 40% methanol: 60% H_2O to ensure purity and resolution. Data was analyzed by determining peak area with Beckman System Gold software on an IBM compatible personal computer. Umbelliferone was tested before and after 2x recrystallization with millipore grade H_2O and toluene on TLC and HPLC. The melting point of the resulting white needles was confirmed with a thermostat controlled stage under a dissecting microscope. As no difference in HPLC analysis, TLC analysis or melting point could be obtained from supplied and recrystallized umbelliferone, supplied umbelliferone given a purity rating of 99.9% from Fluka was used for all biological work.

Characterization of umbelliferone was confirmed via nuclear magnetic resonance (^1H and ^{13}C NMR) and mass spectrometry. NMR of umbelliferone was performed by Dr. G.A. Poulton and Mrs. C. Greenwood on a Perkin-Elmer R32 and Bruker AMX360 NMR spectrometer in the University of Victoria Chemistry Department. Mass spectrometry of umbelliferone and umbelliferone irradiated with NUV was performed by Dr. D. McGillivray at the University of Victoria Chemistry Department on a gas-chromatogram mass spectrum solid probe run at 150°C to 250°C at a rate of 10°C/min. A SE54 nonpolar column with helium as the mobile phase was used.

Both supplied umbelliferone samples' and later irradiated umbelliferone samples' absorption spectra were characterized on a Beckman DU - 64 spectrophotometer. Samples were run at 20 µg/ml in HPLC grade methanol and in phosphate buffer with a 1 cm

pathlength. Scan speed was set at 500 nm/min. and upper absorption limit was set at 2.0.

Fluorescence spectra of supplied umbelliferone were taken on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at different pH's: 6.0, 6.6, 7.0, 7.4, 7.8, 8.0, and 8.2 at 2 different excitation wavelengths of 330 nm and 367 nm for the protonated and unprotonated forms respectively. (Slits 2/2 nm). These tests were done to confirm the spectral behavior of the compound published by Fink and Koehler, (1970) and to later relate it to different biological assays. Umbelliferone was tested at ≈ 0.1 µg/ml in phosphate buffer and data plotted using a Perkin-Elmer 7300 computer.

Biological Assays

a) Lethal photosensitization of *E. coli B/s-1* and *E. coli B/r WP2 try-*

This test was originally designed as a quick ultrasenstive bioassay for detection of photosensitizing compounds (Ashwood-Smith et al, 1983). *Escherichia coli B/s-1* (*rec+, exr-, hcr-*) is a DNA repair deficient bacterium that is highly sensitive to DNA alkylations. This test was done to screen umbelliferone for photobiological activity and then repeated with a wild type DNA repair efficient bacterium, *E. coli B/r WP2 try-* (*rec+, exr+, hcr+*), for comparison of photoactivity.

Umbelliferone was tested in an ultrasensitive photobioassay described by Ashwood-Smith et al., (1983; 1986). Umbelliferone was prepared as 2 mg/ml in methanol as standard and was spotted in various concentrations on TLC plates using 5 or 10 µl Acupipettes™. A minimum concentration for sensitizing activity was established for both repair deficient and wild type *E. coli*. TLC plates were developed in 2D as previously described and were then allowed to dry in the dark in the fume hood for 2 - 3 hrs to remove all solvent traces prior to use. The compounds were detected with UV and the bright blue fluorescent spot marked lightly with pencil. On a test

TLC, this spot was also scraped, extracted with methanol, filtered with a 0.2 μm syringe filter and injected into reverse phase HPLC system to reconfirm compound identity via retention time to ensure that the above procedure did not alter the compound.

Agar plates and soft agar overlay were made as follows-- TNA agar: 10 g tryptone, 8 g NaCl, 15 g Bacto-agar, 1 g glucose and 1 litre dH_2O was autoclaved and poured into square 10 cm x 10 cm petri plates. The overlay was prepared by dissolving and autoclaving 10 g of bacto-tryptone, 6 g bacto-agar, and 5 g of KCl in 1 litre of dH_2O. Agar plates were dried in a sterile laminar flow hood if wet prior to use. TLC plates were pressed onto agar plates and compounds allowed to infuse into the agar for 30 mins. TLC's were then removed and agar plates were ready to receive the overlay.

Bacterial cultures were originally obtained from Dr. B. A. Bridges from the University of Sussex (U.K.) and are described extensively in Bridges et al., (1969). 5 ml of brain heart infusion broth was inoculated with a colony of bacteria in early morning and allowed to grow till approximately 10^8 cells/ml (approx. 5 hrs) with frequent aeration at 37°C. 4 ml of soft agar overlay was dispensed into sterile tubes and kept at 45°C.

100 μl of bacterial suspension was pipetted into overlay tubes and quickly vortexed and poured onto agar plates. When the agar set, plates were irradiated in a closed chamber at a distance of 9 cm from 2 parallel black light bulbs (GEC F20T12-BLB) emitting 13.4 Joules/ m^2/ second (310 - 380 nm, measured by chemical actinometry using ferri-oxalate with a Gaussian distribution round a peak at 350 nm (Ashwood-Smith et al., 1977 and 1983). All irradiations with NUV described in this paper were performed with this light source as described unless otherwise stated. Plates were irradiated for 2 hrs. while dark controls remained covered in a 37°C incubator. All plates were then incubated at 37°C overnight and growth inhibition zones marked and measured with a cm ruler. Dark

control plates allowed distinction between toxicity and photosensitivity.

Umbelliferone crystals pre-irradiated with NUV, sunlight, and fluorescent lab lights on glass and on TLC were also tested for photobiological activity using the above methods and are described in greater detail later in irradiated umbelliferone materials and methods section.

b) Survival curve of *E. coli B/r WP2 try* $^-$

After establishment of photosensitizing activity in the above procedure, an attempt was made to quantify the amount of lethal photosensitization in wild type *E. coli*. Methods used were identical to that of Ashwood-Smith et al., (1982) and were done as a comparison of photoactivity to other coumarins already tested with this same procedure.

15 ml of BHIB (Scott laboratory) was inoculated with one colony of *E. coli B/r WP2 try* $^-$ and grown overnight at 37°C with aeration. 0.1 ml of bacterial suspension was subcultured into 10 ml fresh broth in early morning and grown until the density reached approximately 5 x 10^8 cells/ml. (approximately 4 - 5 hrs.) The resulting suspension was then filtered with a 0.2 µm millipore filter and the bacteria washed twice with 10 ml phosphate buffer (pH 7.2). Bacteria were then resuspended in 10 ml buffer and dilutions made until the density reached 2 x 10^7 cells/ml. For all the following biological tests, umbelliferone standards were made up fresh at least biweekly in 95% ethanol and stored in dark in freezer at -20°C. 5 and 8 mg/ml standards were made and the appropriate one used to give no higher than 1.5% ethanol final content.

Appropriate volumes of standard were added to bacterial suspension to give a final concentration of 40, 60 and 75 µg/ml in separate experiments. A 1.5% ethanol bacterial suspension was used

with each experiment as a negative control. Compounds were mixed with suspensions and allowed to sit for 10 mins. prior to irradiation. Three ml of bacterial/compound suspension was placed in a 10 ml beaker on ice and NUV irradiated under previously described system at a distance of 9 cm. 100 µl of suspension was taken out at 0 time for a dark control and then at 5, 10, or 15 mins. intervals depending upon total irradiation time. The shortest irradiation time was 25 mins. while the longest was 70 mins. (20.1-56.28 kJ/m^2) The 100 µl sample taken out at various intervals was diluted 1:10 four times to give a final concentration of 2×10^3 cells/ml and then 100 µl of this dilution was plated onto nutrient agar plates. Plates were made in triplicate and grown overnight at 37°C. Resultant colonies were counted and plotted to give a % survival curve versus NUV irradiation dose.

This same procedure was done in 10 ml glass bubblers with a) air and b) nitrogen. These bubblers, made by the glass blowing laboratory in the UVic Chemistry Department, resemble 10 ml beakers except the bottom is a dense glass frit with an elongated nozzle for attachment of a hose. These experiments were done to investigate the role of air or nitrogen in the photosensitizing mechanism and the procedure was carried out as described above except that air or nitrogen was bubbled through the suspensions during irradiations. Appropriate controls were done alongside and data was analyzed as above.

As inconclusive results were obtained from the above procedures, this same procedure was then done via irradiation on agar rather than in phosphate buffer. 100 µl of approximately 4×10^3 cells/ml suspension was spread onto TNA plates with varying concentrations of umbelliferone incorporated into the base agar. Concentrations tested were 40 and 75 µg/ml and plates were irradiated for 0, 60, 70, 80 and 120 mins. All plates were done in triplicate and 8-MOP at 40 µg/ml and 1.5% ethanol were used for controls. Plates were incubated overnight at 37°C and resultant surviving colonies were

counted. Data was analyzed using a Student's t-test and a percent survival curve plotted.

c) Frameshift mutagenesis of *E. coli lac⁻,z,thiamine⁻* ND160

As the photosensitizing ability of umbelliferone was finally established in the previous experiments, the mechanism of action was investigated next. Intercalation into DNA was tested by the ability to frame-shift mutate the *lac z* gene in *E. coli*. Frameshift mutagenesis studies in the dark were conducted as described by Bridges and Mottershead (1977), and by Ashwood-Smith (1978) and is a well substantiated test for intercalation ability (Ames et al., 1973; Ames and Whitfield, 1966). Bacteria used, *E. coli* K-12 ND160, *lac⁻ z, thiamine⁻*, are described in Clarke and Wade (1975) and were originally donated by Dr. B.A. Bridges. This bacterium has a reading frame-shift mutation in the *lac z* gene making it unable to use lactose as an energy source. However, intercalation by a compound can alter the DNA secondary and tertiary structure, thereby altering the reading frame and reverting bacteria from *lac⁻* to *lac⁺* (Pindur et al., 1993). This intercalation can also cause base insertions or deletions during DNA replication which could also cause a frame-shift mutation to revert bacteria to *lac⁺* (Pindur et al., 1993).

Reagent recipes are recorded in the appendix. Umbelliferone and 8-MOP as a positive control were incorporated into the base agar of petri plates at time of pouring to give a final concentration of 40 µg/ml for 8-MOP and 40, 60, 75, 100, 125, and 150 µg/ml for umbelliferone. All base agar used for frame-shift tests was minimal media supplemented with lactose and thiamine. Base agar plates with a final 1.5% ethanol concentration were used as negative controls for each experiment.

Bacterial identity was reconfirmed with ampicillin resistance tests and lactose/thiamine requirements using standard procedures. The experimental procedure in summary is as follows: bacteria

were grown overnight with aeration in BHIB supplemented with glucose (2% w/v) and thiamine (0.25 μg/ml) at 37°C and subcultured in early morning (0.1 ml into 10 ml same broth). Bacteria were allowed to attain logarithmic growth (approximately 4 hrs.) and harvested via a 0.2 μm millipore filter. Bacteria were washed twice with 10 ml phosphate buffer and resuspended in the same to give a final density of approximately 10^8 cells/ml. A series of 8 one in ten dilutions were made (dilution 0 being overnight culture) and 100 μl of dilution 1 plated in triplicate under red lights for each test compound: 8-MOP, umbelliferone, and ethanol. 100 μl of dilution 6, 7, and 8 were also plated on nutrient agar to determine cell viability. Plates were incubated in the dark at 37°C for 2 days. Mutant colonies were counted and data analyzed via Student's t-test. Background mutation frequency on control plates was also calculated to ensure a low background rate of mutation.

d) Frameshift Mutagenesis with *Salmonella typhimurium* TA98 in the Ames test

Following the above testing with *E. coli lac⁻*, umbelliferone's ability to frameshift in another biological system was tested. The Ames test is a simple mutagenicity test that screens compounds by incubation with *Salmonella typhimurium* tester strains with/without liver microsomal enzyme conversion. These methods are extensively described in Maron et al., (1983). TA98 has a frameshift mutation in a repetitive GC sequence inactivating the histidinol dehydrogenase gene and has two additional mutations, *uvrb⁻*, *rfa⁻* ,that greatly increase its sensitivity to mutagens by reducing its DNA repair capabilities and increasing its membrance permeability to compounds. TA98 also contains a biotin synthesis mutation and an ampicillin resistance plasmid. All strain characteristics were confirmed via outlined procedures in Maron et al., (1983). A frameshift in the above described gene reverts histidine requiring (*his⁻*) bacteria to *his⁺*.

The method in summary is as follows: 15 ml of Oxoid nutrient broth No. 2 was inoculated with one colony and grown overnight with aeration. 0.1 ml of culture was subcultured into 10 ml fresh broth in early morning and grown to a density of 1-2 x 10^9 cells/ml. S9 mix was made from the supernatant fraction of a centrifuged, Aroclor-induced rat liver homogenate combined with $MgCl_2$, KCl, glucose-6-phosphate, phosphate buffer, NADP, and NaH_2P0_4. Microsomal enzymes in the liver homogenate have been shown to activate compounds into carcinogens and are one method used to test compound activity as a result of metabolism. Minimal-glucose base agar petri plates were made as per Maron et al., (1983) and dried in a laminar flow hood if wet.

A soft agar overlay as above was supplemented with a final concentration of 0.05 mM histidine/biotin solution to ensure that a background bacterial growth resulted. This was done to allow a few rounds of replication to allow mutation expression and to safeguard against toxicity. Agar was dispensed into 2 ml tubes and umbelliferone was incorporated into test overlays at a concentration of 50 and 150 µg/ml of overlay (< 1.5% ethanol content). Umbelliferone was tested for biological activity with and without S9 activation. Benzo(a) pyrene was tested +/- S9 activation as a positive control at a concentration of 0.5 µg/ml of overlay and 1.5% ethanol was added to negative control overlay tubes.

0.1 ml of TA98 bacterial broth was added to each overlay tube. S9 mix was kept on ice until immediately prior to addition of bacteria. 0.5 ml of S9 mix was placed in tubes designated for S9 metabolism and then tubes vortexed and poured over agar plates. 0.5 ml of phosphate buffer was placed in tubes minus S9 and those tubes were vortexed and poured. Plates were incubated at 37°C for 48 hrs. and number of histidine revertants scored. Data was analyzed via Student's t-test.

e) Lethal photosensitization of Chinese hamster ovary cells

After confirmation of photobiological activity in 2 bacterial assays, a mammalian cell line was tested. Because there are many differences between eukaryotic and prokaryotic cells, and metabolism within mammalian cells has been shown to significantly alter photobiological activity, an in vitro test was done with Chinese hamster ovary cells (Ashwood-Smith et al., 1992 and 1982). Materials and methods follow that of Natarajan et al., (1981) and Ashwood-Smith et al., (1980). Serially propagated Chinese hamster ovary fibroblasts designated as Puck's clone A (CHO cells) were grown in McCoy's 5a medium (Terry Fox Laboratory) supplemented with 15% fetal bovine serum and streptomycin/penicillin. Monolayers of cells were grown at 37°C in Falcon 70 ml sterile plastic tissue culture flasks. Cells were harvested prior to confluence by pipetting off medium followed with 0.25% (w/v) trypsin treatment for 5 mins. Enzyme action was stopped with serum supplemented McCoy's 5a medium and cells were harvested via centrifugation at 75xg for 3 mins. The supernatant was pipetted off and cells washed twice with the above medium minus serum. The pellet was resuspended in 5 ml fresh medium minus serum and density obtained by cell counts using a Neubaeur haemocytometer.

Appropriate numbers of cells were seeded into flasks containing 8 ml fresh serum supplemented medium to obtain approximately 200 colonies after the irradiation procedure. Numbers of seeded cells were calculated taking into account predicted survival of treatment and a 60% cloning efficiency. For example, if the expected kill rate was 90% for 5 mins. irradiation at 40 µg/ml umbelliferone, approximately 3000 cells would be seeded to end up with approximately 200 surviving cells (for a 66% cloning efficiency). Three flasks were seeded for each test concentration and each exposure time. Flasks were gassed after seeding with 5% $C0_2$ and incubated for 4 hrs. at 37°C to allow cells to attach.

8-MOP was used as a positive control at 40 μg/ml in McCoy's 5a medium minus serum and 1.5% ethanol was used for a negative control. Umbelliferone was tested at 40 μg/ml and 160 μg/ml in McCoy's minus serum. All compound standards were made in 95% ethanol and made at a concentration which resulted in <1.5% ethanol content when added to medium. Irradiation doses for 8-MOP were 0, 20, 40 and 60 secs. while doses ranged from 0 to 25 mins. for umbelliferone (13.4 Joules/m^2/sec.) (see fig. 28 for dosages). Three flasks of seeded cells received no treatment whatsoever to serve as another negative control and stayed in the incubator during the entire experimental procedure. These flasks were used to obtain baseline cloning efficiency.

After 4 hrs., the medium was pipetted off flasks and saved. 5 ml of test solution warmed to 37°C: 8-MOP, umbelliferone or ethanol was added gently to flasks and flasks were gassed again with 5% $C0_2$. Cells were then allowed to sit for 10 mins. at room temperature to allow diffusion of compounds into them.

Flasks were put on a tray, covered and then slipped under NUV lights already described in method 2 at a distance of 9 cm. The cover was removed for the required irradiation time and then put back on prior to removal from lights. Dark controls for the 8-MOP, umbelliferone, and 1.5% ethanol treatment stayed on the laboratory bench for the longest period of time until all irradiations were completed.

Test solutions were then removed and discarded and cells were washed with two washes of 5 ml McCoy's 5a medium minus serum for 5 mins. each. Previously saved medium was replaced and flasks gassed with 5% $C0_2$ and incubated at 37°C for 10 days. Medium was then discarded and cells rinsed twice with 0.85% saline followed by increasing concentrations of methanol, and then stained with 2 ml undiluted Giemsa. Resultant colonies were then counted and survival plotted as a function of NUV irradiation dose for each test compound.

Any observed differences between 1.5% ethanol and umbelliferone were tested for significance with a Student's t-test.

f) Inactivation of the ampicillin resistance gene in pTZ18R

Because of conflicting results with biological activity in bacterial versus mammalian cells and in different frameshift detection systems, a simple biological assay was developed to confirm that photoreaction could be occurring with DNA and not by any other mechanism. Plasmid DNA was irradiated in vitro solely in the presence of millipore water, DMSO, and the test compound. Photoreaction was tested by the ability of the intact ampicillin gene in the plasmid to confer ampicillin resistance to bacteria upon transformation.

Transformation of bacteria by the uptake and integration of plasmid DNA has been documented extensively (Micklos and Freyer, 1990; Sambrook et al., 1989; Mandel and Higa, 1970). Transformation in our assay was exhibited by the transcription of the ampicillin resistance gene of the pTZ18R plasmid in Stratagene Epicurian Coli SURE™ cells. Inactivation/mutation of this gene, for example via photoalkylation by coumarins, would cause a decrease in the number of transformants.

8-MOP, a known DNA photomutagen, was tested against coriandrin (a furanocoumarin depicted in fig. 32 and known to form DNA monoadducts only) and umbelliferone in this in vitro plasmid DNA irradiation assay. The objective of this experiment was to show conclusively that the two test compounds react with naked DNA as no other biological targets were present during the assay. Coriandrin, a novel furanocoumarin, described extensively in Ceska et al., 1988 and Ashwood-Smith et al., 1989, was found to be a photomutagen, and photosensitizing agent in bacterial/mammalian cells, and photoclastogen in CHO cells. Coriandrin's results will only be discussed in reference to umbelliferone's photobiological activity.

Plasmid isolation was carried out as per Qiagen plasmid maxiprep Tip 100™ protocol and transformation procedures as per Stratagene Epicurian Coli™ competent cells protocol. (All reagent recipes are attached in appendix). All methods are summarized in brief and the reader is asked to refer to above protocols for details. Stratagene Epicurian Coli SURE™ cells containing pTZ18R plasmid were a gift from Dr. Wil Hintz in the Biology Department at the University of Victoria. One colony was streaked on an ampicillin 2X YT plate and grown overnight at 37°C. In early morning, 100 ml of 2X YT broth was inoculated with one colony from the plate and grown 24 hrs. at 37°C with shaking. Cells from culture were pelleted by centrifugation at 3000 g and the supernatant discarded. Cells were lysed with lysis buffer for 5 mins. and then mixed with SDS/NaOH by gentle inversion and iced for 10 mins.

An ice cold solution made from 60 ml of 5 M potassium acetate and 11.5 ml glacial acetic acid made up to 100 ml final vol. with dH_2O was then added and the tube was then iced for another 10 mins. The mixture was then centrifuged and supernatant saved and filtered through a fine silk mesh. 3 ml QBT buffer was added to a Qiagen Tip 100 column for equilibration which was not allowed to dry between solvents. Filtered supernatant was then poured through column and then column washed with 10 ml QC buffer. DNA was harvested in a final elution step with 5 ml QF buffer. DNA was then precipitated with 0.7 volumes of ice cold isopropanol in freezer overnight. DNA was pelleted via high speed centrifugation (16,000 rpm) and supernatant discarded. DNA was then washed with 70% ethanol and pelleted again. Supernatant was discarded and the pellet was dried in the fumehood. Quantity, purity and integrity of DNA was checked on a 1% agarose gel run with ethidium bromide in the running buffer and OD 260/280 nm on a Beckman SPD 400 spectrophotometer. DNA was made into a 1 µg/µl stock with millipore water and kept in the dark in the freezer at -20°C.

Different quantities of non-irradiated plasmid DNA were tested to establish a DNA transformation efficiency curve. This was done to determine the linear portion of the curve to select an amount of DNA to be used in later irradiations to ensure that a decrease in intact DNA would be detected in a decrease in transformants (Micklos and Freyer, 1990; Mandel and Higa, 1970). 6 µl of a 1 µg/µl stock plasmid DNA was diluted 1:100 and 300 µl of this used to check the concentration at OD 260 nm. Diluted DNA stock was then serially diluted in order that 5 µl of the final diluted stock would give the required amount of plasmid DNA for each experiment. A constant 5 µl volume was chosen to eliminate errors produced by volume differences in transformation suspensions. For example, if 4.0 ng, 1.0 ng, and 0.4 ng DNA were to be tested for transformation ability, 5 µl of a 0.8 ng, 0.2 ng, and 0.08 ng/µl stock were used.

Epicurian Coli SURE ™ competent cells from Stratagene were purchased and used for transformation experiments. These patented cells which are engineered for high transformation efficiency, are also deficient in DNA rearrangements and DNA repair. (Genotype and address for further inquiries to Stratagene are enclosed in the appendix). The transformation protocol was the same for non-irradiated and irradiated plasmid. Six transformation conditions could be tested in each experiment-- i.e. 6 different concentrations of DNA for transformation efficiency experiments or 6 different dosages of a test compound + irradiation.

A 240 µl vial of SURE ™ cells was thawed on ice just prior to using and 3.4 µl of *B*-mercaptoethanol added and finger vortexed to mix. 40 µl of this suspension were then aliquoted into each of 6 prechilled 1.5 ml Eppendorf tubes. Tubes were iced for 10 mins. with gentle swirling to mix every 2 mins. 5 µl of each plasmid DNA dilution was added to each tube and microfuged. Tubes were then iced for 30 mins., heat shocked at 42°C for 45 secs. and then iced for 2 mins. further. 1 ml of 2X YT broth (at 37°C) was then added to each tube and incubated at 37°C for 1 hr. with occasional shaking.

After incubation, bacterial cultures were plated on 2X YT ampicillin plates that were nondiluted, diluted 1:10 and diluted 1:100 for each test condition. Bacterial dilutions were made with 2X YT broth. $1:10^4$ and $1:10^5$ dilutions were also plated on nutrient agar plates for cell viability for at least one of the 6 tubes. The number of transformed bacterial colonies (exhibiting ampicillin resistance) were counted after 20 hrs. of incubation at 37°C. Transforming efficiency was calculated per μg of DNA used. Using lot #1 of SURE ™ cells, amount of plasmid DNA used versus number of bacterial transformants was plotted through 4 trials of transformations using different quantities of plasmid. From this graph, it was determined that 4.0 ng of DNA was in the linear portion of the curve and was selected for later irradiation experiments. This graph was reconfirmed through 2 trials for the 2nd lot of obtained SURE ™ cells.

For irradiation of plasmid DNA with test compounds, 6 μl of the 1 μg/μl stock plasmid DNA was diluted 1:100 and 300 μl of this used to check concentration at OD 260 nm. The remaining 300 μl was diluted to 600 μl with millipore H_2O and vortexed. 95 μl was dispensed into a chilled 1.5 ml Eppendorf tube and 1 μl DMSO added as a negative control. Remaining DNA was usually divided between into a 300 μl aliquot and a 200 μl aliquot to test 2 separate compounds. Compounds were made up in DMSO (either 500 μg/ml or 1.5 mg/ml standard) and were added to aliquotted DNA giving a final 1% (v/v) DMSO concentration. A final coriandrin/ 8-MOP concentration of 5 μg/ml and umbelliferone of either 5 or 15 μg/ml was used. A nonirradiated control was done for each compound.

95 μl of each test solution was pipetted onto a piece of clean parafilm fitted into a 35 mm petri dish. Parafilm was chosen because the liquid sits as a sphere on the surface providing maximum surface area for irradiation. This dish was placed in a larger 100 mm petri dish containing a small quantity of ice and covered with the corresponding petri dish lid. This entire dish was

placed on a large tray containing ice. The tray was placed at a distance of 9 cm away from NUV light source for irradiation. This setup was designed to limit evaporation during irradiation and ice was replenished as necessary.

Compound + DNA without irradiation was left on parafilm until all irradiations were completed. After irradiation times, the ball of liquid was taken up with a Gilson P200 micropipettor and measured. Millipore H_2O was added to make up the final volume to 94 µl to compensate for evaporation. Only 1 or 2 µl was required for even the 2 hour irradiation periods. The DNA ball was then diluted with millipore H_2O to give a final concentration of 0.8 ng/µl. 5 µl of this to give 4.0 ng was used to transform SURE ™ cells as per protocol above. Depending on the experiment, the DMSO control without compound was either not irradiated to give a baseline number of transformants or irradiated for the longest irradiation time in the experiment to ensure irradiation was having no effect on the plasmid DNA.

Plates were done in triplicate for each test compound and irradiation time. Number of ampicillin resistant transformants for each treatment were counted and Student's t-test done on the mean to establish statistical significance. SURE ™ cells were also plated for viability on nutrient agar for at least the control and one test compound to ensure that numbers of cells in each transformation were approximately the same: 100 µl of a $1:10^4$ and $1:10^5$ dilution were plated in triplicate on nutrient agar plates.

Irradiation of umbelliferone

Although umbelliferone was purchased pure, small traces of impurity were found on both TLC and HPLC. Various attempts were made to purify it further but achieved little success. It was finally discovered that umbelliferone was being converted into other products in the presence of light. Because of photobiological activity of umbelliferone in some tests but not in others, it was

possible that umbelliferone itself was not photoactive but that its photoproducts were. 5-geranoxypsoralen was found in our laboratory to be photobiologically inactive in itself but formed very active breakdown products when degraded by light or metabolized by mammalian cells (Ashwood-Smith et al., 1992).

To investigate the biological activity of umbelliferone and its photoproducts, all in vivo biological tests were repeated with irradiated umbelliferone except for the Ames test and lethal photosensitization of CHO cells. For the TLC bioassay, 26 µg of umbelliferone was irradiated on TLC for 2 hrs. prior to development and imprinting and visualization of any photoproducts was made under 254 nm and circled with pencil. This was compared to non-irradiated umbelliferone for photoactivity against *E. coli B/s-1*.

Umbelliferone was also spotted on TLC and then irradiated for varying times under NUV light prior to development. Umbelliferone was irradiated on TLC for 0, 1, 2, 5, 8, 10, 12, 15, 18, 20, 22, 25 and 30 mins. and then developed in 1D to monitor rate of production of photoproducts. These TLC plates were also used in bioassay to screen for activity. An equal amount of umbelliferone was also tested concurrently in the standard assay method but instead of 2 hr. irradiation with bacteria, was tested with 0, 10, 20, 40, 80, 120 and 160 mins. of NUV light to compare rate of production of breakdown products with photoactivity. (All NUV light irradiation dosage= 13.4 Joules/m^2/sec).

26 µg of umbelliferone was also spotted and then irradiated as above for 2 hrs of NUV light and then developed in 1 direction. Produced compounds were visualized with UV light at 254 nm, marked with pencil, and then irradiated again prior to development in the 2nd direction. This allowed visualization of any interconversion or further breakdown of photoproducts and these plates were also tested for photoactivity.

To further investigate which compound was active, umbelliferone or its photoproducts, 0.1, 1 and 5 μg of umbelliferone were spotted and developed on each of 5 TLC's. 0.1 μg was an approximation made from spotting 1 μl of a 1 in 10 dilution of a 1 μg/μl stock. All TLC's were imprinted on agar plates and following, 3 agar plates were irradiated under NUV light for 2 hrs. while the other two were kept in the dark at equal temperature. Bacterial overlays were poured on all 5 plates after irradiation and bioassay performed on 4 plates. One pre-irradiated umbelliferone plate was incubated in the dark as a control to ensure that formed photoproducts were not toxic. Resultant inhibition zones were measured with a ruler and compared.

TLC bioassay was also repeated with irradiated umbelliferone on glass to test if breakdown was facilitated by binding to silica. Umbelliferone was evaporated into a thin film on a watchglass and irradiated for 2 hrs. This was then taken up in methanol and spotted in varying quantities on TLC and compared with non-irradiated umbelliferone. Both were also tested in bioassay for activity.

One final possibility tested was to investigate if umbelliferone was being chemically changed to a photoactive substance as a result of binding to TLC. Supplied umbelliferone was dissolved in millipore H_2O and approximately 25 μg of a 200 μg/ml solution was slowly absorbed through spotting onto the center of a TNA agar plate. This received the standard *E. coli* $B/_{s-1}$ overlay for bioassay after 30 mins. diffusion time.

Survival of *E. coli B/r try*$^-$ was tested with umbelliferone irradiated in phosphate buffer (pH 7.2) for 2 hrs. under NUV light prior to addition of bacteria in an already described procedure. 100 μl bacterial suspensions were then taken at various irradiation times and the experiment carried out as per outlined protocol. Survival of *E. coli B/r try*$^-$ was also tested with umbelliferone irradiated in phosphate buffer in sunlight for 8 hrs. All described irradiations in sunlight were done in July, 1993 in Victoria, BC

starting at 8:30 am in the morning. A dark umbelliferone control was always tested alongside each of the above tests.

Frameshift mutagenesis of *E. coli lac*$^-$ was tested by incorporating umbelliferone into the base agar at 60 and 100 μg/ml (< 1.5% ethanol). Six plates were made for each concentration and 3 were irradiated under NUV light for 2 hrs. while remaining were covered. Frameshift experiment was done as outlined and results between irradiated umbelliferone and normal umbelliferone compared with Student's t-test analysis. 8-MOP and 1.5% ethanol controls were performed as outlined.

To show conclusively that umbelliferone was photodegrading, a series of experiments were performed. Equal amounts of umbelliferone were dissolved to saturation in methanol, ethanol, or millipore H_2O and irradiated at a distance of 9 cm away from NUV light source. All liquid layers in 20 ml beakers were < 3 mm in depth and occasionally stirred during their 2 hrs. irradiation. Irradiated crystals on glass and non-irradiated umbelliferone were used as controls. After irradiations, all compounds were evaporated to dryness and immediately dissolved in methanol to equal concentrations. Equal amounts were spotted on TLC for 1D development for comparison. All samples were also further dissolved to 20 μg/ml in methanol and absorption at 325 nm taken on Beckman DU-64 spectrophotometer as previously outlined. Samples irradiated in methanol and ethanol were analyzed by mass spectrometry for photoproducts.

It was observed on TLC that even after recrystallization, standard umbelliferone left a very small amount of yellow product at the origin and also at an Rf value of 0.30-0.33 using hexane/acetone 1:1. (Umbelliferone had an Rf value of 0.88). In an attempt to harvest these impurities for characterization, 200 μg of a 5 mg/ml umbelliferone in methanol standard was streaked in a line on TLC and developed in 1D in the dark in hexane/acetone 1:1. Under red light, both yellow impurities were separately scraped and

extracted in 90% methanol/10% water. Extracted compounds were cleaned by filtration through a 0.2 μm millipore filter and evaporated to dryness in the fume hood. Extracted compounds were then streaked separately on another TLC and redeveloped as before.

After TLC's were dried and visualized with UV, the same TLC's were then redeveloped in the same direction two times more to ensure that all umbelliferone traces were removed from the yellow bands. Harvesting of isolated impurities were to be done as above and it was hoped that enough yellow compounds could be obtained for identification.

Umbelliferone was also spotted on 3 TLC's and either irradiated in sunlight or fluorescent room lights or covered in the dark to test for photoproducts. 30 μg of umbelliferone was spotted on each of 3 TLC's and then irradiated on a window with direct sun, left on a lab bench under room lights, and left on a lab bench covered for 2 consecutive days from 8:30 am till 6:30 pm. All described irradiations with sunlight were done in July, 1993 in Victoria, BC. Spots were then developed in the first direction with hexane/acetone 1:1 and in the second direction in methanol/ water 80:1. Photoproducts were visualized with UV light and also tested for photoactivity using TLC bioassay against *E. coli B/s* $_{-1}$.

Fluorescence and absorbance spectra were also taken of irradiated umbelliferone in phosphate buffer solution at two pH's in already described method to show the photodegradation of the compound and to characterize new products. Umbelliferone was irradiated in NUV light at a distance of 9 cm for 0, 25, and 80 mins. in CHO tissue culture flasks at a concentration of 40 μg/ml in phosphate buffer at pH 6.8 and 7.8 (made from a 5 mg/ml 95% ethanol standard/ final ethanol concentration 0.8%). Control flasks of 0.8% ethanol in phosphate buffer were also irradiated for the same amount of times. All flasks were gently agitated during irradiations. Total volume of solution was 5 ml in each flask and

fluid depth no greater than 3 mm. 100 µl samples were taken out at each time and diluted 1:1000 with phosphate buffer (final [ ] approximately 0.04 µg/ml) and stored in dark and on ice for fluorescence spectra.

300 µl in an exact repeat experiment the following day were taken out at each time for absorbance spectra and diluted to a final concentration of 10 µg/ml. Absorbance and fluorescence spectra were done immediately on solutions following irradiations and solutions receiving no irradiation were done last.

Results

Characterization

The identity of supplied umbelliferone was verified through NMR and melting point. NMR was verified by Dr. G. A. Poulton and Mrs. C. Greenwood at the Chemistry Dept. at the University of Victoria and was confirmed as published. Before and after recrystallization twice with millipore H_2O or toluene, resultant white needles had a melting point of 225-228 ^oC characteristic of umbelliferone (Budavari, 1989). Mass spectrometry confirmed a molecular weight of 162.2. Absorption spectra and fluorescence spectra of umbelliferone were confirmed as published in the literature (Murray et al., 1982; Fink and Koehler, 1970) and are enclosed in the appendix (figs. 48-50).

The purity of umbelliferone was checked with silica TLC plates with and without fluorescent indicator before and after recrystallization in various 1D and 2D solvent systems. Only 1 plate is shown in fig. 5 as hexane/acetone 1:1 was the best system for separation and no additional impurities could be found using other methods. When greater than 30 μg of compound was spotted, TLC analysis showed that a very minute quantity of yellow product stayed at the origin and at an Rf value of 0.30-0.33. Umbelliferone had an Rf value of 0.88.

As shown by figs. 5 and 6, even after repeated crystallizations in water or toluene, umbelliferone showed trace quantities of impurity on TLC and HPLC. On reverse phase HPLC, trace impurities had a shorter retention time than umbelliferone: 3.59 compared to 3.89 mins. The amounts of impurity were 0.36 area% before and 0.41 area% after recrystallization. The melting point of umbelliferone, however, was not depressed compared to the literature. The yellow compound at Rf 0.30 showed up sporadically depending on quantity of umbelliferone spotted and time left on TLC prior to development. The bright blue spot with an Rf value of 0.88 was reverified as

Figure 5. 1D Silica TLC with hexane/acetone 1:1 of 30 µg umbelliferone a) before and b) after being recrystallized twice with millipore H_2O. The following symbols stand for the color of fluorescence of the compound under UV: Y=yellow, B=blue.
Umbelliferone had an Rf value of 0.88 while yellow impurities had an Rf value of 0.00 and 0.30-0.33.

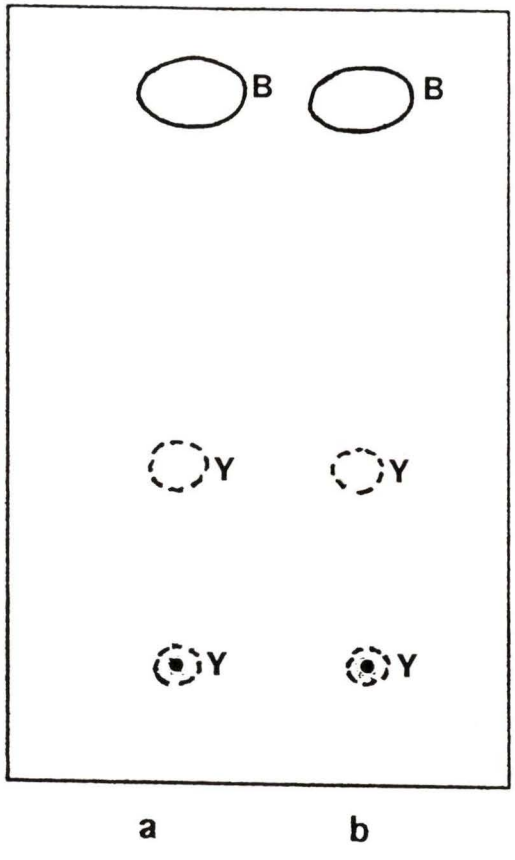
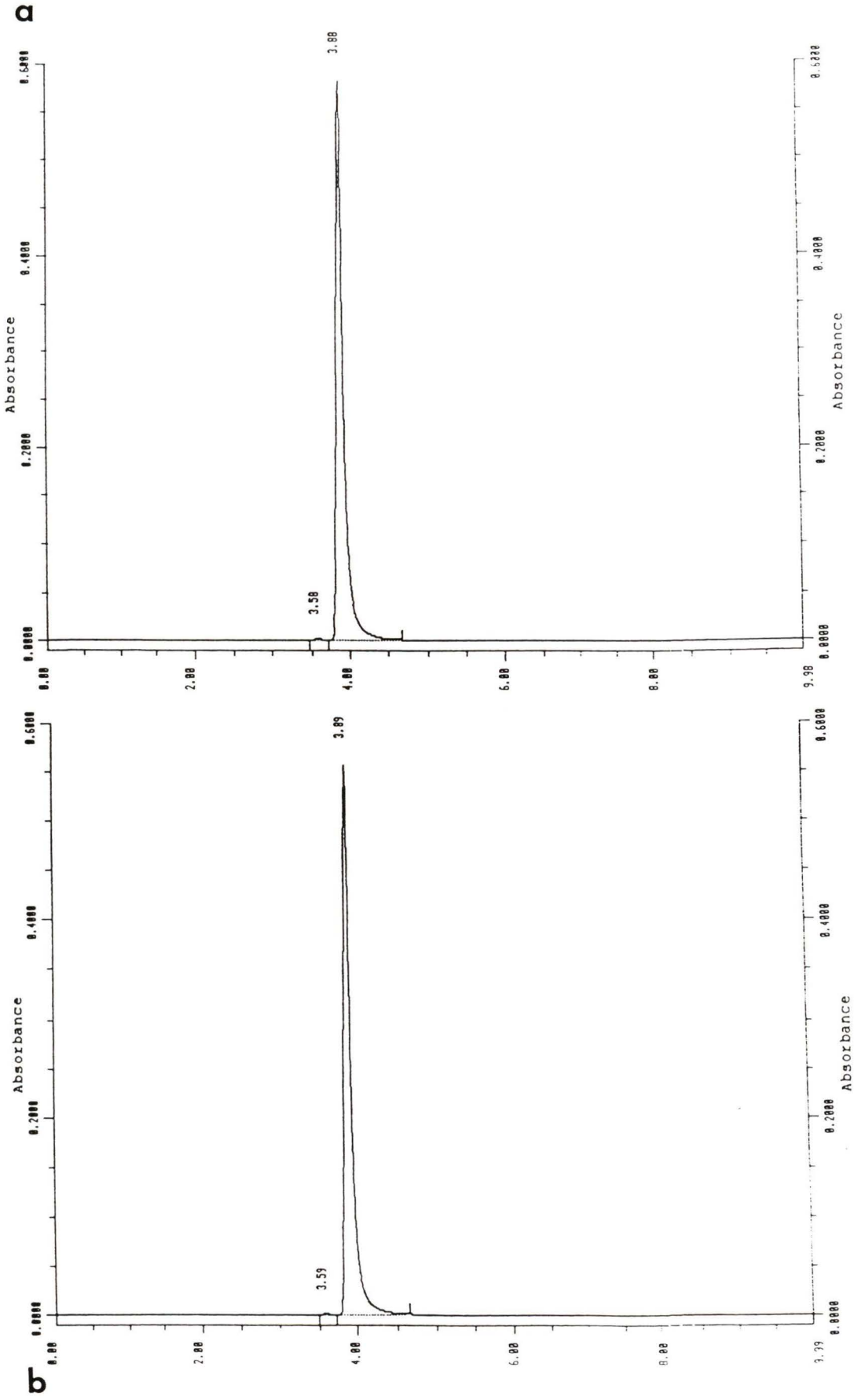

B
B
Y
Y
Y
Y
a
b

Figure 6. Reverse phase high performance liquid chromatogram of umbelliferone before and after being twice recrystallized with millipore H_2O. Umbelliferone was dissolved at 10 µg/ml mobile phase and chromatogram was obtained using a Beckman 126 pump, Beckman 166 detector and System Gold integration software. Samples were run on 4.6 mm x 25 cm ultrasphere ODS analytical column with 5 µm pore size and using a flow rate of 1 ml/min. The mobile phase used was 80% CH_3CN and 20% water and detection wavelength set at 254 nm.
Umbelliferone Rt before recrystallization = 3.88/ after recrystallization Rt = 3.89
impurity before recrystallization: Rt= 3.58, 0.36 area%
impurity after recrystallization: Rt = 3.59, 0.41 area%


a
Absorbance
3.88
3.58
b
Absorbance
3.89
3.59

umbelliferone by scraping of TLC and extraction with methanol and testing by both HPLC retention time and melting point after extraction.

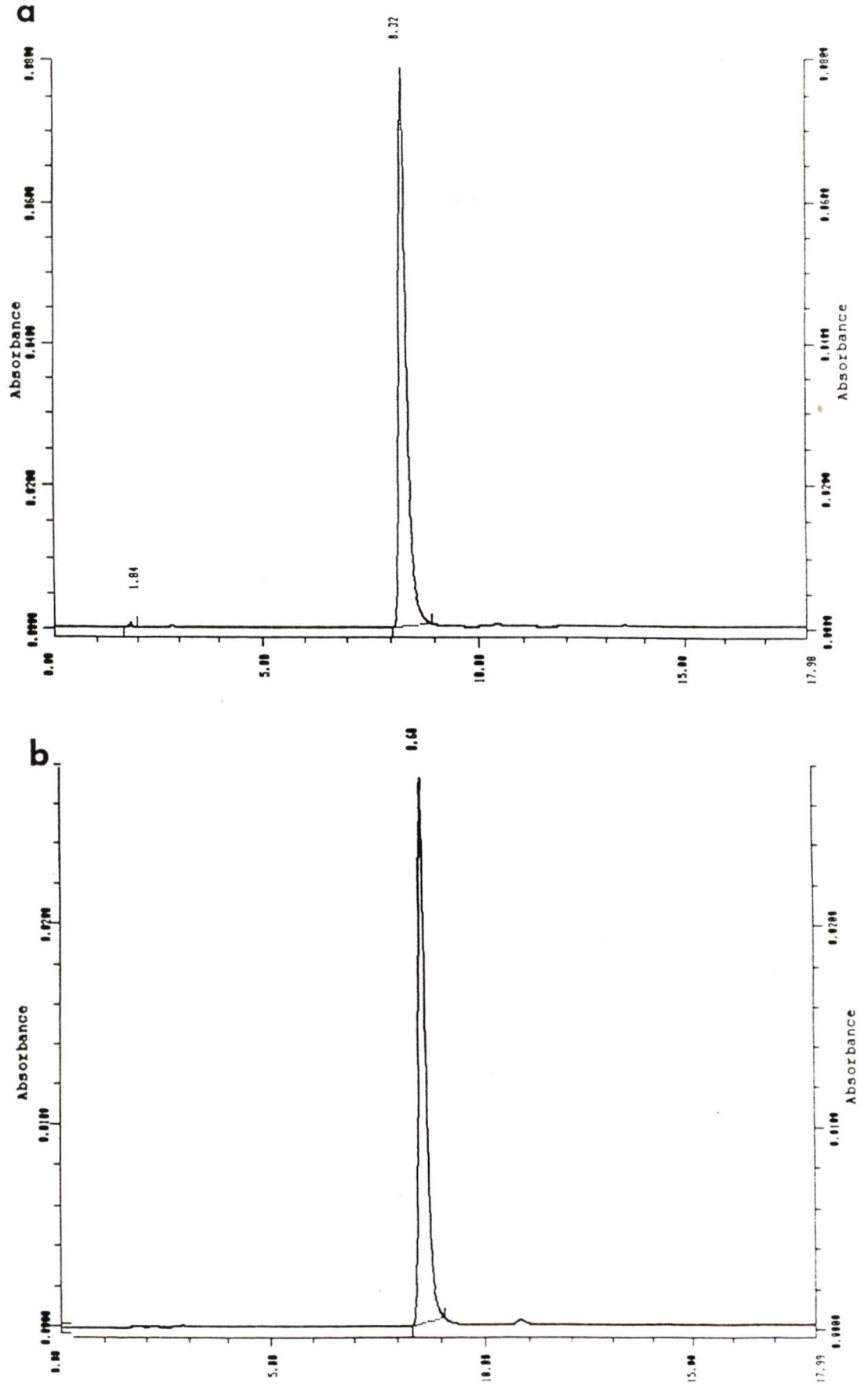
As shown by fig. 6, HPLC analysis showed that supplied and recrystallized umbelliferone had no difference in retention time or purity: 99.7%. Umbelliferone was tested for purity using various other solvent compositions from 95% CH_3CN/5% water to 40% CH_3CN/60% water and also 70% methanol/30% water to 40% methanol/60% water. Various detector wavelengths were also used to ensure purity: 240, 254, and 325 nm. A sampling of those chromatograms are given in fig. 7. No other impurities were noted except for an occasional baseline fluctuation at approximately 10.8 mins. at 254 nm using 60% water/40% CH_3CN. Various gas chromatograms performed by Dr. D. McGillivray prior to mass spectrometry also showed no significant impurities. One example is shown in fig. 8.

It was found that if umbelliferone crystals were left exposed to lab lights, the amount of impurity in TLC analysis increased. TLC plates irradiated in sun and fluorescent lab lights versus a dark control also produced much more yellow product at the origin and sometimes slightly more at Rf 0.30-0.33 when developed in hexane/acetone 1:1. Products at the origin were separated by 2DTLC with hexane/acetone 1:1 in the first direction (solvent I) and methanol/water 80:1 (solvent II) in the second as shown by fig. 46 on page 147.

As shown by fig. 46a, standard umbelliferone had an Rf value of 0.88 in solvent I and 1.0 in solvent II. Impurities left at origin in solvent I had an Rf value of 0.92 (yellow) and 0.84 (blue) respectively in solvent II. As shown by fig. 46b, sun irradiated umbelliferone had the usual umbelliferone and its impurities but also a new blue product with an Rf of 0.14 in I and 0.96 in II and a new dark yellow product with an Rf of 0.00 in I and 0.96 in II. The

Figure 7. Reverse phase high performance liquid chromatograms of umbelliferone using different wavelengths and mobile phases to check purity. Samples were run at 20 µg/ml of mobile phase. A Beckman 126 pump, 166 detector and System Gold integration software were used. Samples were run on a 4.6 mm x 25 cm ultrasphere ODS analytical column with 5 µm pore size and a) 240 nm detection and 60% HPLC water and 40% HPLC methanol b) 254 nm detection using above solvent and c) 325 nm detection with 70% methanol/30% water. All flow rates were 1 ml/min.

a) injection artifact Rt= 1.84 mins., 0.38 area %
   umbelliferone Rt= 8.32 mins., 99.62 area%
b) umbelliferone Rt= 8.60 mins., 100 area%
c) umbelliferone Rt= 3.56 mins., 99.39 area%
   possible impurity or overloading Rt= 3.95 mins., 0.60 area%


a
Absorbance
1.84
8.32
5.00
10.00
15.00
17.98
Absorbance
b
Absorbance
8.68
5.00
10.00
15.00
17.99
Absorbance

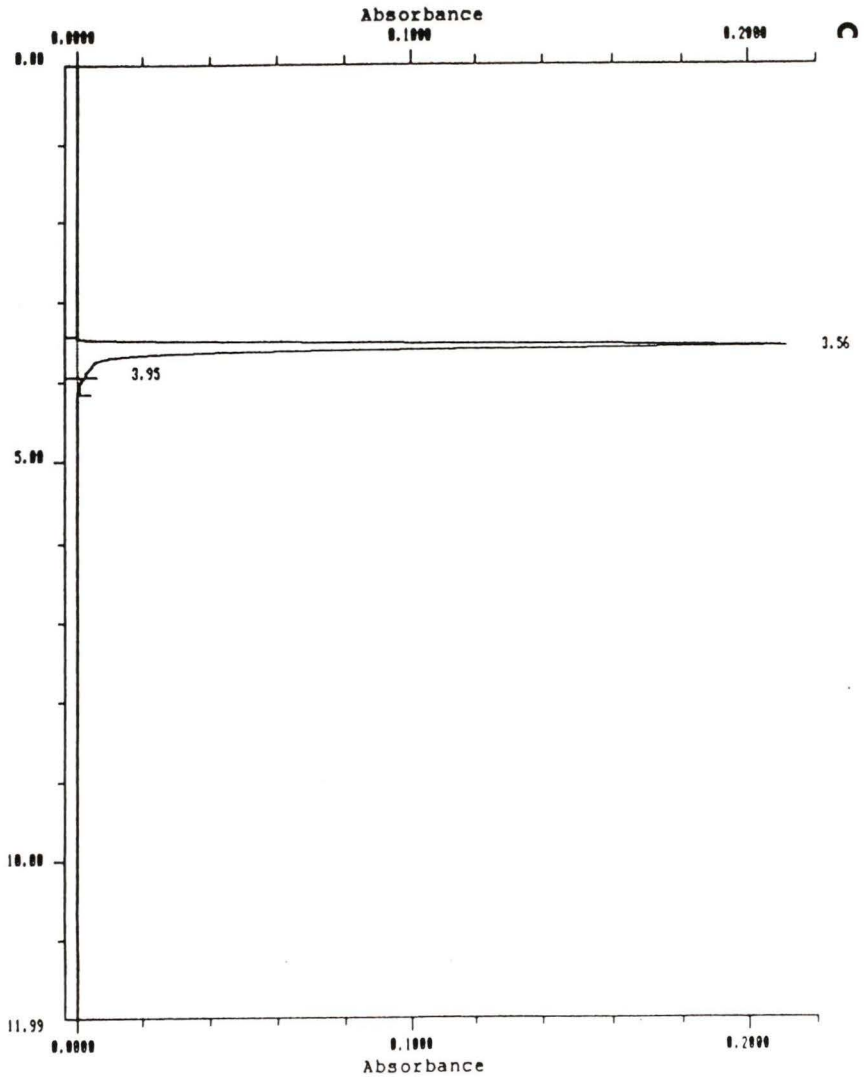
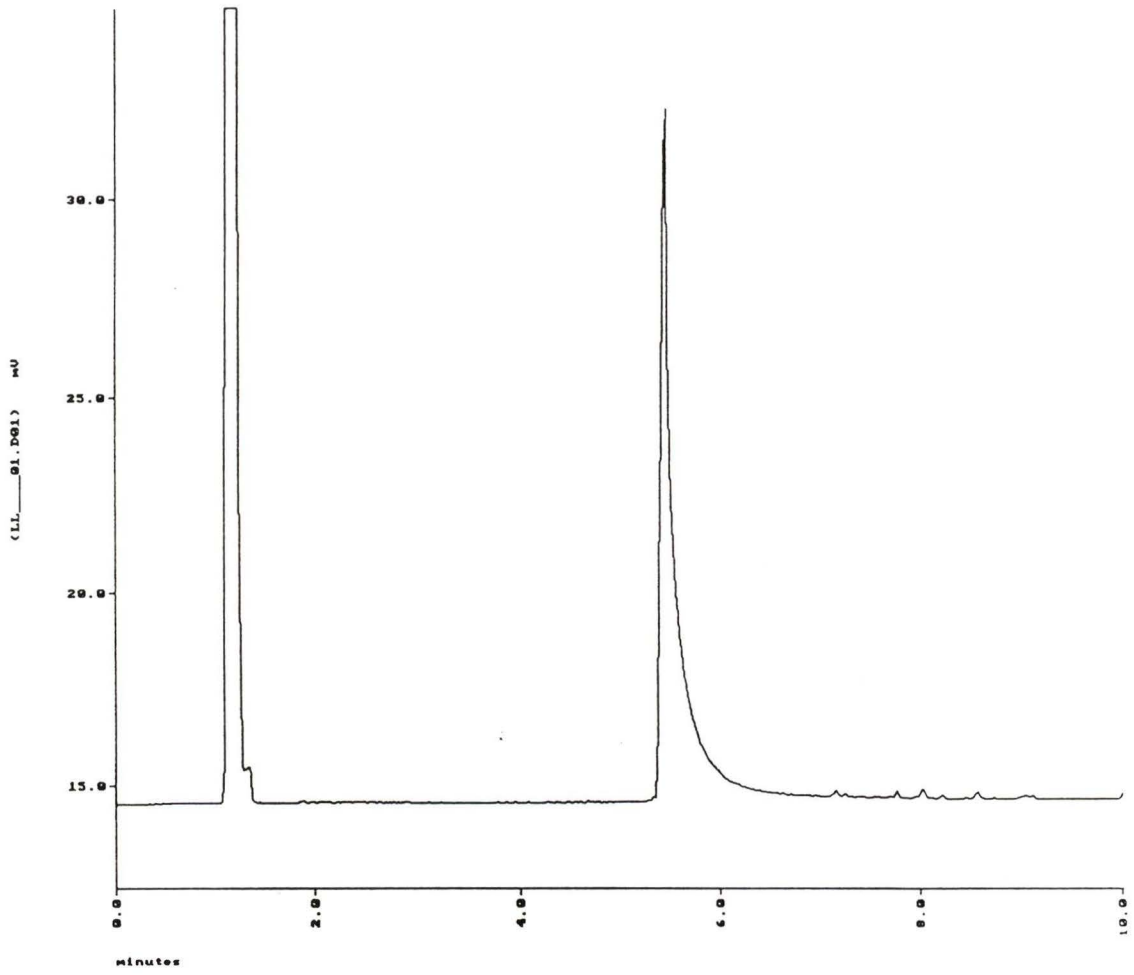

Absorbance
0.0000
0.1000
0.2000
0.00
5.00
10.00
11.99
3.56
3.95
Absorbance

Figure 8. Gas chromatogram of umbelliferone using 150°C - 250°C at a rate of 10°C/min. on a SE54 nonpolar column with helium as the mobile phase.


(LL____01.D01) mV
30.0
25.0
20.0
15.0
0.0
2.0
4.0
6.0
8.0
10.0
minutes

blue and yellow impurities present on standard umbelliferone were present in greater quantities on the sun irradiated compound. Fluorescent light irradiated umbelliferone also had the new dark yellow product with an Rf of 0.00 in I and 0.95-0.96 in II but no new blue one at 0.14 in I (see fig. 46c). Impurities present in the standard were again present in greater quantities in fluorescent light irradiated sample.

As stated in materials and methods, an attempt was made to isolate the two yellow bands of Rf 0.00 and 0.30 through developing 200 μg of compound on 1D TLC followed by extraction and rechromatographing in the dark (fig. 9a). However, as shown by fig. 9b, when the yellow impurity at Rf 0.30 was first run on TLC, a significant portion now had an Rf value of 0.88. After cutting away the TLC at the arrow and redeveloping for final removal of umbelliferone traces, the yellow bands gradually disappeared while additional blue bands appeared (see fig. 9b). The impurity at the origin acted in a similar fashion and a figure demonstrating this is given in the paragraph following. These yellow products thus appeared to convert back to umbelliferone and therefore it was not possible to identify them.

A 2D TLC of 20 μg of standard umbelliferone using hexane/acetone 1:1 is shown in fig. 10a. Irradiation of 20 μg umbelliferone with NUV light at the origin on TLC increased the quantity of yellow impurity at the origin and also catalyzed formation of new ones as shown by fig. 10b and c. As shown by fig. 10a and b, there was impurity left at the origin after each development with the same solvent. The impurity left at the first origin converted to umbelliferone during the second development in the other direction in both figures. After NUV irradiation, as shown by fig. 10b, new photoproducts formed with Rf values of 0.72-0.74 (blue), 0.62 (blue) and 0.54 (yellow) as seen under UV. These photoproducts from NUV irradiation shown in fig. 10b also seemed unstable as some would convert from one to another if reirradiated between the 1st and 2nd development on TLC as shown by fig. 10c.

Figure 9. Isolation of umbelliferone impurity at the origin and Rf 0.30 by TLC and hexane/acetone 1:1 development. a) 200 µg of standard umbelliferone was streaked and developed on TLC plate. The symbols indicate the color of fluorescence of the compound under UV: y=yellow, B=blue, y + B= yellow and blue. Impurity at the origin was yellow with a blue ring encircling it. The impurity at the origin and Rf 0.30 were scraped and extracted with methanol and then b) respotted separately on TLC plates and developed once again to the top of the plate. The yellow impurity previously at Rf 0.30 when developed showed that the majority of the compound was now blue with an Rf value equal to that of umbelliferone (0.88). After cutting the TLC at the arrow to remove origin impurities, further development of the TLC to the solvent front marked as s2 showed further conversion to a blue product with an Rf of 0.88. The impurity at the origin showed a similar behaviour but data is not shown.

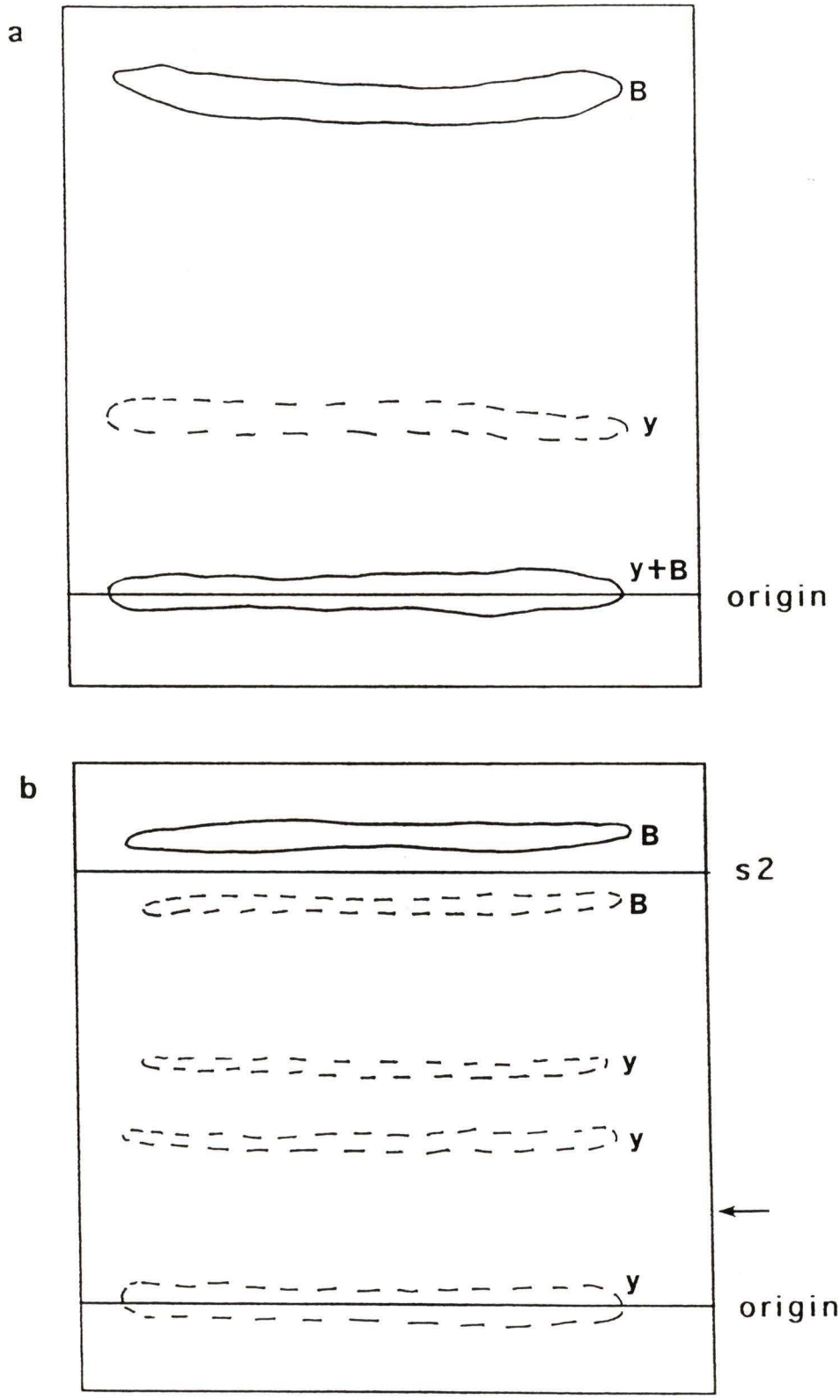
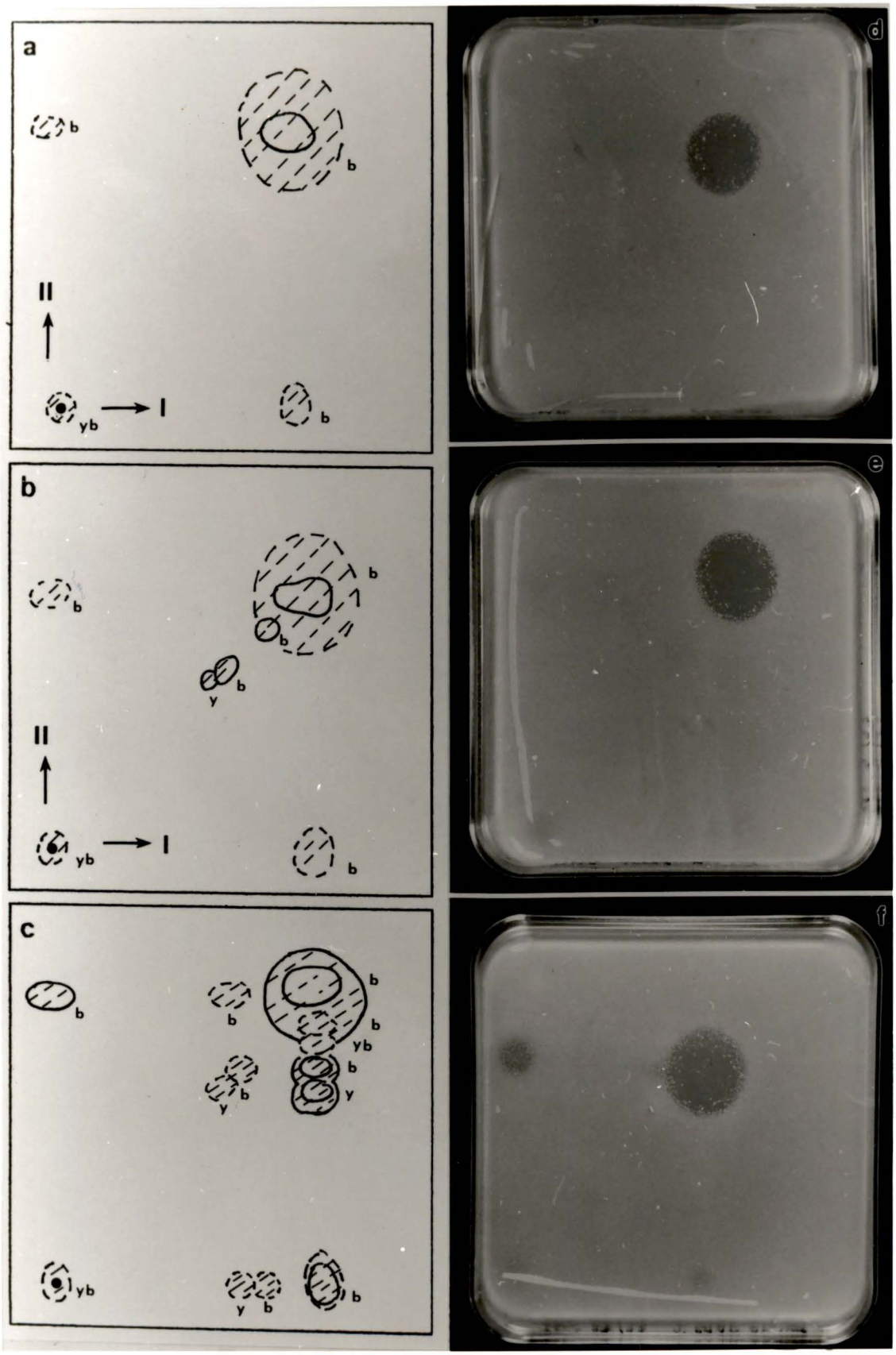

a
B
y
y+B
origin
b
B
s2
B
y
y
y
origin

Figure 10. 2D TLC's of 20 μg of umbelliferone using Merck Silica plates without fluorescent indicator a) before and b) after crystals irradiated on TLC with NUV for 9.65 x 10^4 Joules/m^2. c) 26 μg of umbelliferone irradiated on the origin for above quantity of NUV and then developed in 1 direction and then irradiated again as above prior to development in 2nd direction. Hexane/acetone 1:1 was used in both directions (I and II) for all plates. Two to five photoproducts were shown on TLC after irradiation. After NUV, as shown by fig. 10b, new photoproducts formed with Rf values of 0.72-0.74 (blue), 0.62 (blue) and 0.55 (yellow). Similar photoproducts formed at 0.73 (yellow/blue), 0.67 (blue), and 0.58 (yellow) in fig. 10c and a new product at 0.79 (blue). Umbelliferone had an Rf value of 0.88. The new photoproducts from NUV irradiation also seemed unstable as some would convert from one to another if reirradiated after development in the first direction as shown by fig. 10c. All plates were then used in TLC/*E. coli B/s-1* photobioassay to test for photosensitization and inhibition zones are represented by clear zones on agar in d, e and f and also by shading on a, b and c. Inhibition zones over all photoproducts were very faint as shown by photographs with only strong inhibition zones present over umbelliferone.


a
b
b
II
yb
I
b
b
b
b
b
b
y
II
yb
I
b
c
b
b
b
b
yb
b
y
y
b
yb
b
b
d
e
f

Photoproducts formed with similar Rf values at 0.73 (yellow/blue), 0.67 (blue), and 0.58 (yellow) in fig.10c and a new photoproduct at 0.79 (blue). These products are further discussed in irradiated umbelliferone results section.

Silica has been known to catalyze the chemical conversion of many compounds, so umbelliferone was also irradiated under NUV on glass and in solution. Irradiated umbelliferone samples in methanol, ethanol, water, and on glass under NUV light also appeared to form photodegradative products (see fig. 44 on page 143). These products also seemed unstable as they would again convert from one another in the process of a 2D TLC in the dark (see fig. 45 on page 145). These experiments showed conclusively that umbelliferone was forming photoproducts which differed depending on the conditions of irradiation. Absorption at 325 nm was also done for umbelliferone irradiated in different solutions in an attempt to quantify the amount of conversion. These results are discussed in more detail under irradiated umbelliferone results section. Because of the above results confirming photodegradation and the lack of differences on HPLC or TLC between recrystallized and supplied umbelliferone, umbelliferone supplied from Fluka and given a purity rating of 99.9% was used for all biological assays.

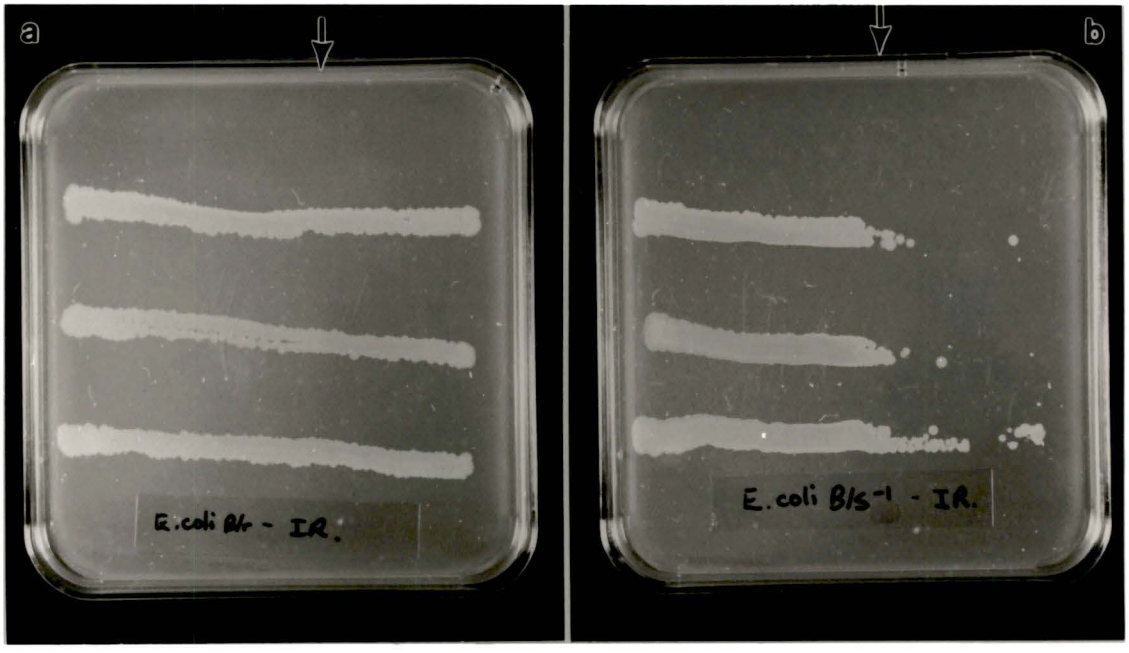
Biological Assays

a) Lethal photosensitization of *E. coli B/s-1* and *E. coli B/r WP2 try*$^-$

The identity of wild type and DNA-repair-deficient bacteria wsd confirmed by streaking logarithmic cultures on TNA plates and irradiating half the plate with 254 nm for 335 Joules. *E. coli B/s-1* could not repair induced thymidine dimers and no growth was evident on that side of the plate in contrast to wild type *E. coli B/r* (see fig. 11).

Figures 10a and d shows that 20 µg of umbelliferone tested in a photobioassay against *E. coli B/s-1* producing a large clear

Figure 11. a) Wild type *E. coli B/r* and b) DNA repair deficient type *E. coli B/s-1* identities were confirmed by streaking mid-logarithmic cultures on TNA plates and irradiating the right half of the plate (marked with an arrow) with UV light (254 nm) for 335 Joules/m^2. Plates were then grown overnight at 37°C. No growth was evident on the irradiated side of the *E. coli B/s-1* plate in contrast to wild type *E. coli B/r*.


a
E.coli B/r - IR.
b
E.coli B/s-1 - IR.

inhibition zone not present in a dark control. Umbelliferone was found to be a very active lethal photosensitizing agent against *E. coli B/s-1* with a minimal detectable limit of approximately 0.1 µg/ spot. This zone was fairly faint and hazy and a clear photosensitization zone was not produced until approximately 0.3 µg/ spot was used. (Quantities were approximate as they were made from spotting 1 µl of 1:10 dilutions of a 1 or 3 µg/µl stock.) Figures 10a, b, and c also show binding of impurities at origins on both directions of 2DTLC and some interconversion of the compound at the initial origin back to a compound with an identical Rf value of umbelliferone. Very faint inhibition zones were present over all photoproducts; however, they did not show clearly in the photograph. Despite large quantities of umbelliferone used, the border of the inhibition zone was always ragged and colonies could always be found growing within the outer portion of the zone. This was in sharp comparison to inhibition zones produced by highly active photoactive compounds such as 8-MOP as shown by fig. 12.

This same test was used against an overlay of *E. coli B/r WP2 try⁻*, but a discernible inhibition zone could not be detected until 20 µg/ spot was used (see fig. 13). 80 µg/ spot was used as the upper limit of the test, but the inhibition zone was still hazy and only within the area closest to the compound imprint as shown by fig. 13. The agar overlay had an average depth of 3 mm and as shown by fig.13, the photosensitization zone increased in depth from 1/3 to 2/3 to about 9/10 of the overlay depth for 20, 50 and 80 µg of umbelliferone used. These zones also increased diameter with increasing amounts of compound from 0.85 cm for 20 µg to 1.1 cm for 80 µg. This again was in high contrast to the clear zones produced by 8-MOP in both *E. coli* strains and by umbelliferone in *E. coli B/s-1* (see fig. 12). All of the following TLC bioassays described use the DNA repair deficient bacterium, *E. coli B/s-1*.

As 5-geranoxypsoralen was found in our laboratory to only be photoactive through its photoproducts, both irradiated and nonirradiated umbelliferone were investigated for photoactivity. To

Figure 12. Photobioassay with *E. coli B/s-1* a) on approximately 25 µg of umbelliferone dissolved in millipore water and absorbed onto the center of a TNA plate. b) Dark control of bioassay with *E. coli B/r WP2 try-* with 80 µg of umbelliferone absorbed on a Silica plate and then absorbed onto TNA plates. c) photobioassay with *E. coli B/s-1* and 0.5 and 1 µg of 8-methoxypsoralen spotted onto a TLC plate and then absorbed onto a TNA plate. d) photobioassay with *E. coli B/r WP2 try-* with 80 µg of umbelliferone absorbed on a Silica plate and then absorbed onto TNA plates. Bacterial cultures were grown to mid-logarithmic stage prior to testing. All NUV irradiations were performed x 96.48 kJ/ m^2.

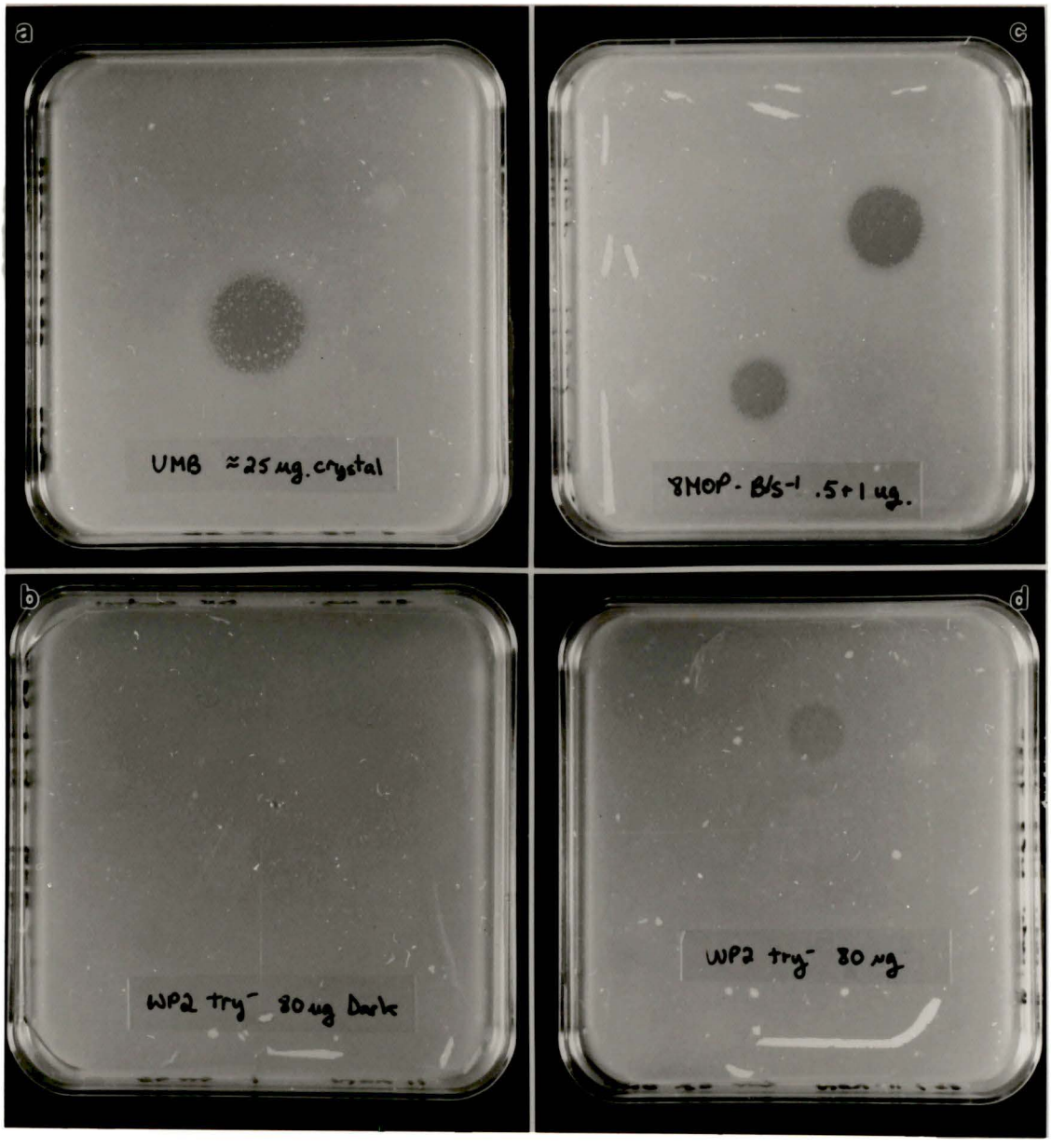
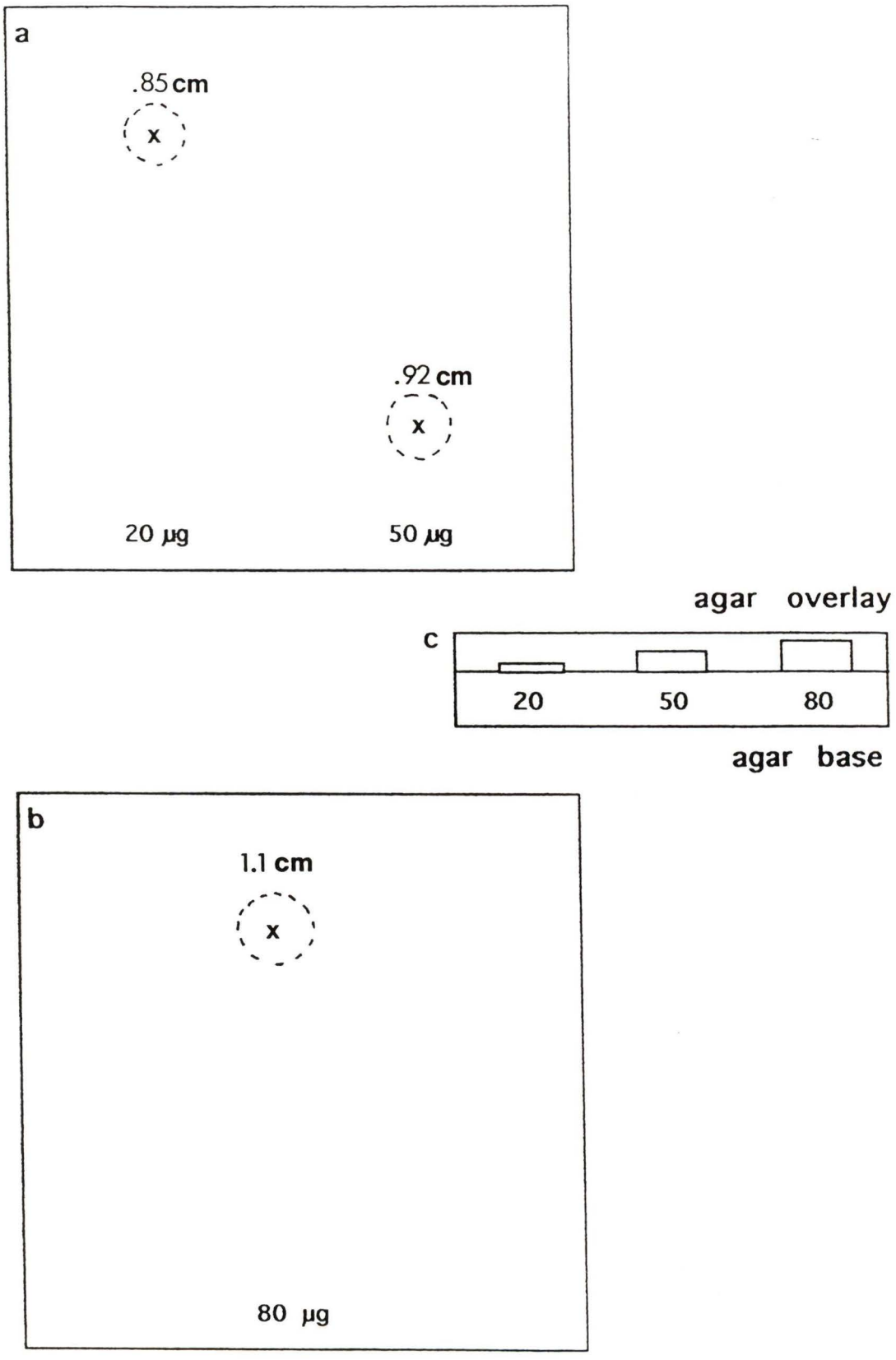

a
UMB ≈25 ug. crystal
c
8MOP - B/S-1 .5+1 ug.
b
WP2 try⁻ 80 ug Dark
d
WP2 try⁻ 80 ug

Figure 13. TLC bioassay with *E. coli B/r* with 20, 50 and 80 µg of umbelliferone spotted on centers of areas marked with an x but not developed on TLC. Inhibition zones are marked with a dashed perimeter and diameters measured with a ruler. Inhibition zones were not clear as they were only present in the agar overlay closest to the compound as shown by c). Inhibition zone width increased with increasing amount of compound but never extended the entire width of bacterial agar overlay.


a
.85 cm
x
.92 cm
x
20 µg
50 µg
agar overlay
c
20
50
80
agar base
b
1.1 cm
x
80 µg

test if umbelliferone's breakdown products were active, umbelliferone was irradiated on TLC and then developed in 2D. All photoproducts were marked with pencil as seen with UV and the TLC then tested on bioassay. Umbelliferone produced its usual photosensitization zone and all photoproducts produced a very faint hazy zone as shown by figs. 10 c, e and f. However, it was not clear whether umbelliferone was photoactive or whether it was only active through production of other products during the assay.

To investigate this question, the photobioassay was performed for 0, 10, 20, 40, 80, 120 and 160 mins. of NUV irradiation instead of the standard 120 mins. A comparative analysis was performed on equal amounts of umbelliferone NUV irradiated on TLC for 1, 2 , 5, 8, 10, 12, 15, 18, 20, 22, 25, and 30 mins. Significant photobiological activity could be discerned after just 10 mins. of NUV irradiation on bacteria (8.04 kJ/m^2) as shown by shading on fig. 14 but only very faint traces of photoproducts could be found after 5 mins. of irradiation on TLC (4.02 kJ/m^2) (see fig. 15). Slight increases in amount of photoproduct were noted on TLC from 5 to 18 mins. but no discernible increases could be noted from 18 to 30 mins. (14.47 kJ - 24.12 kJ/m^2) and quantity of photoproduct never exceeded 0.5 cm^2 in size. However, the size of the photosensitized zone in the bacterial overlay steadily increased from an area of 0.95 cm^2 at 10 mins. to approximately 2.4 cm^2 at 80 mins. and plateaued from 80 to 160 mins (see fig. 14.) (64.32 -128.64 kJ/m^2). Photoproducts developed from 18 mins. irradiation also produced only faintly discernible inhibition zones.

To further investigate which compound was active, umbelliferone or its photoproducts, 0.1, 1 and 5 µg of umbelliferone were spotted and developed on each of 5 TLC's in 1D as shown by fig. 16. (0.1 µg was an approximation only as it was made from a 1:10 dilution of a 1 µg/µl stock.) All were imprinted on agar plates and following, 3 plates were irradiated under NUV for 2 hrs. (96.48 kJ/m^2) while the other two were kept in the dark at the same temperature. Bacterial

Figure 14. Umbelliferone in standard TLC photobioassay method with *E. coli B/s-1* with increasing irradiation with NUV from 10 mins. to 160 mins. (80.4 to 128.64 kJ/ m^2). Inhibition zone borders are shown by dashed lines. The inhibition zone was present at a) 10 mins. but quite hazy. The zone increased in size and became clearer until it reached a plateau at b) 80 mins. TLC development was done with ethylacetate/chloroform 4:1 in first direction (I) and then followed by hexane/acetone 1:1 in second direction (II).

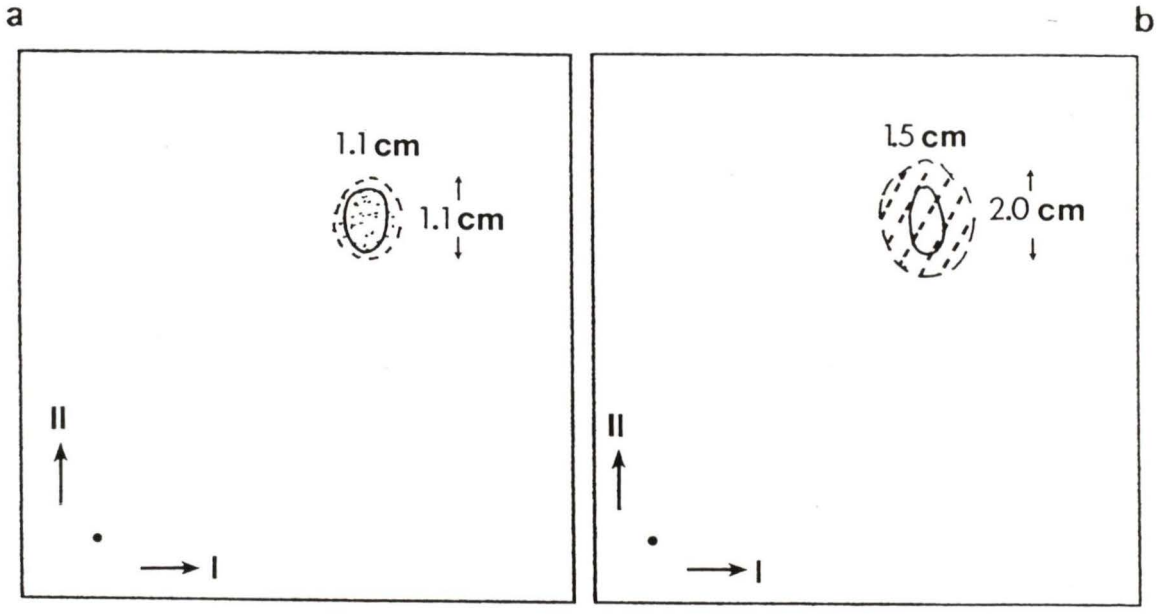
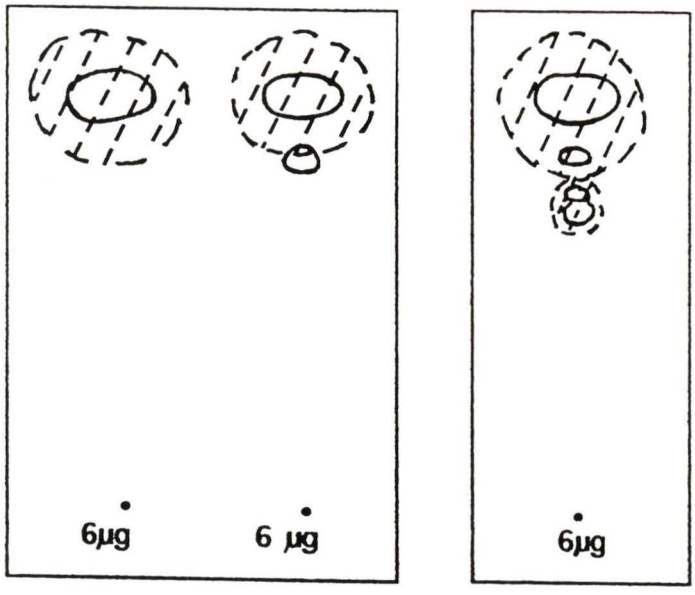

a
1.1 cm
1.1 cm
II
I
b
1.5 cm
2.0 cm
II
I

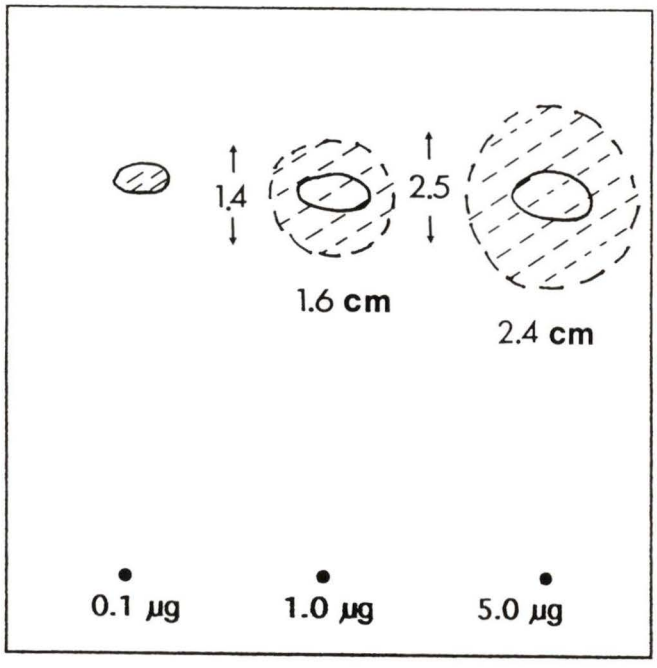
Figure 15. 6 μg of umbelliferone spotted on Silica TLC and subsequently irradiated for 0 - 30 mins. and developed in hexane/acetone 1:1. a) 0 mins., b) 5 mins., and c) 18 mins. No photoproducts were noted until 5 mins. (4.02 kJ/ m^2) with slightly more noticeable products at 18 mins. No increase in quantity of photoproducts occurred between 18 and 30 mins. (14.47 kJ - 24.12 kJ/ m^2). Rf values were as follows: umbelliferone 0.88, yellow/blue photoproduct 0.77, blue photoproduct 0.67 and blue photoproduct 0.63. Colors given were fluorescence as seen under UV. Inhibition zones from subsequent TLC bioassay with *E. coli* *B/s-1* are shaded with dashed lines. Inhibition zones over photoproducts were very faint while zone over umbelliferone was clear except around the perimeter.



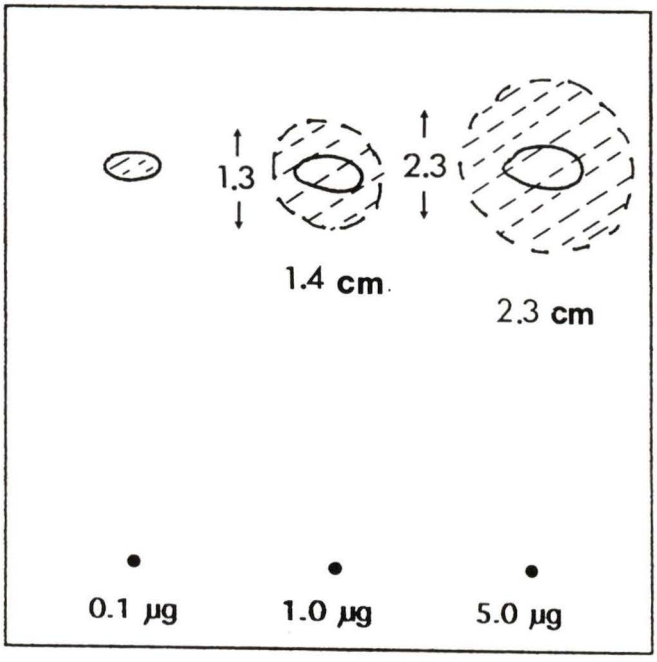
0 mins. 5 mins. 18 mins.

Figure 16. Photobioassay with 0.1, 1 and 5 µg of umbelliferone developed on TLC with *E. coli B/s-1*. (0.1 µg was an approximation only as it was made from a 1:10 dilution of a 1 µg/µl stock.) Umbelliferone was spotted on each of 5 TLC plates and then infused into separate agar plates and 3 were pre-irradiated under NUV x 96.48 kJ/ m^2. All plates then received bacterial overlay followed by irradiation with NUV x 96.48 kJ/ m^2. Inhibition zones, as shown by shaded areas, were measured and compared. Zones produced by pre-irradiated umbelliferone (b) were slightly smaller when measured at the diameter than standard umbelliferone plates (a) by approximately 1-2 mm in both directions but differences were not statistically significant.

a



b

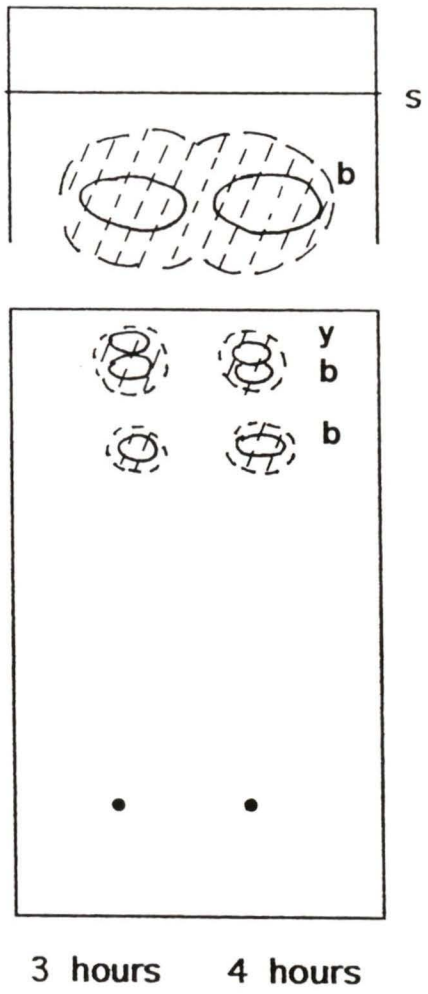


overlays were then poured on all 5 plates and bioassay performed on 4 plates. One pre-irradiated umbelliferone plate was incubated in the dark as a control to ensure that formed photoproducts were not toxic. Resultant inhibition zones were compared and are shown in fig. 16. The dark control was negative for toxicity while the pre-irradiated umbelliferone zones were slightly smaller than non-irradiated umbelliferone as shown by fig. 16. Although pre-irradiated umbelliferone appeared to be, if anything, less photoactive than standard, this difference was not statistically significant.

To test if photoproducts could be active through reconversion into umbelliferone, umbelliferone was spotted on TLC and irradiated for 2 hrs. (see fig. 10c). This was then developed in 1 direction with hexane/acetone 1:1 and then irradiated further for 2 more hrs. This was then developed in the second direction. As seen in fig. 10c, some photoproducts could convert back and forth into one another and back into umbelliferone. A subsequent photobioassay showed all products produced weak photosensitization zones as shown by shaded areas on fig. 10c. Umbelliferone also formed 3 photoproducts when irradiated on glass and not just on TLC. Irradiated crystals for 3 and 4 hrs. were developed by later TLC analysis in the dark which showed 3 new weak photobiologically active spots when tested on bioassay as shown by fig. 17. Inhibition zones are shown by shading.

One final possibility tested was to investigate if umbelliferone were being chemically changed to a photoactive substance as a result of binding to TLC. Supplied umbelliferone was dissolved in millipore H_2O and approximately 25 μg of a 200 μg/ml solution was slowly absorbed through spotting onto the center of a TNA agar plate. This umbelliferone, without silica involvement, also produced a photoinhibition zone as shown by fig. 12a.

Figure 17. Umbelliferone crystals irradiated on glass for 3 and 4 hrs. of NUV (144.72 and 192.96 kJ/ m^2). 20 µg of compound was developed on TLC plates with hexane/acetone 1:1. Rf values were as follows: umbelliferone= 0.88, yellow photoproduct = 0.76, blue photoproduct = 0.72, and blue photoproduct 0.61. Symbols (y) and (B) indicate color of fluorescence as seen under UV. Photobioassay inhibition zones with *E. coli B/s* $_{-1}$ is marked by shading. The TLC was cut and bioassay tested separately on TLC halves to prevent blurring of inhibition zones by compound diffusion. Inhibition zones over photoproducts were very faint. Solvent front is shown by line marked with an s.


S
b
y
b
b
3 hours
4 hours

b) Survival curve of *E. coli B/r WP2 try*$^-$

Survival curves with *E. coli B/r WP2 try*$^-$ in phosphate buffer were disappointing. As shown by fig. 18, no conclusive photosensitization could be produced despite positive results on TLC photobioassay. Concentrations of 40, 60, and 75 μg/ml were used with increasing NUV from 0 to 70 mins. (56.28 kJ/m^2). 60 μg/ml results are not shown as they were similar to 40 μg/ml of umbelliferone. A significant difference between control and test treatments was produced at 70 mins. at 75 μg/ml once when bacteria were irradiated from the bottom of a flat 70 ml tissue culture flask instead of through solution in a 20 ml beaker. This is depicted in fig. 18 but survival for umbelliferone/NUV treated bacteria decreased to only 62.3% of control. Because the standard deviations between plates were low, the difference between control and treatment was $p= 0.004$ on a Student's t-test. Due to this very low photosensitizing effect, bubbling through solution with air or nitrogen was attempted next to increase the lethality of the treatment.

Because of the prior evidence of umbelliferone photodegradation and the possibility that photoproducts could be the active agent, bubbling oxygen or nitrogen through solutions may have catalyzed the formation of these products. Alternately, if umbelliferone sensitized cells through photooxidation effects, air bubbling would have also increased bacteria death. Survival from 60 μg/ml umbelliferone/NUV treated bacteria decreased to 25.6% of control/NUV treated after 56.28 kJ/m^2 of irradiation with air bubbling ($p< 0.001$). Control bacteria irradiated in 1.5% ethanol decreased to 57% when compared to a non-irradiated control whereas bacteria treated with umbelliferone and NUV decreased to 16.4% when compared to nonirradiated. However, these results could not be reproduced when attempted with a higher concentration of umbelliferone as shown by fig. 19.

Figure 18. Survival of *E. coli B/r WP2 try*$^-$ irradiated in phosphate buffer. *E. coli B/r* was grown overnight in nutrient broth to mid-log phase and washed and resuspended in phosphate buffer (0.07 M) to a concentration of approximately 2 x 10^7 cells/ml. Bacteria were irradiated with either a 1.5% ethanol control or 40 or 75 µg/ml of umbelliferone. Bacteria were then diluted until approximately 2 x 10^3 cells/ml and 100 µl plated on nutrient agar plates which were then grown overnight. Neither NUV nor umbelliferone had any lethal effects on their own.

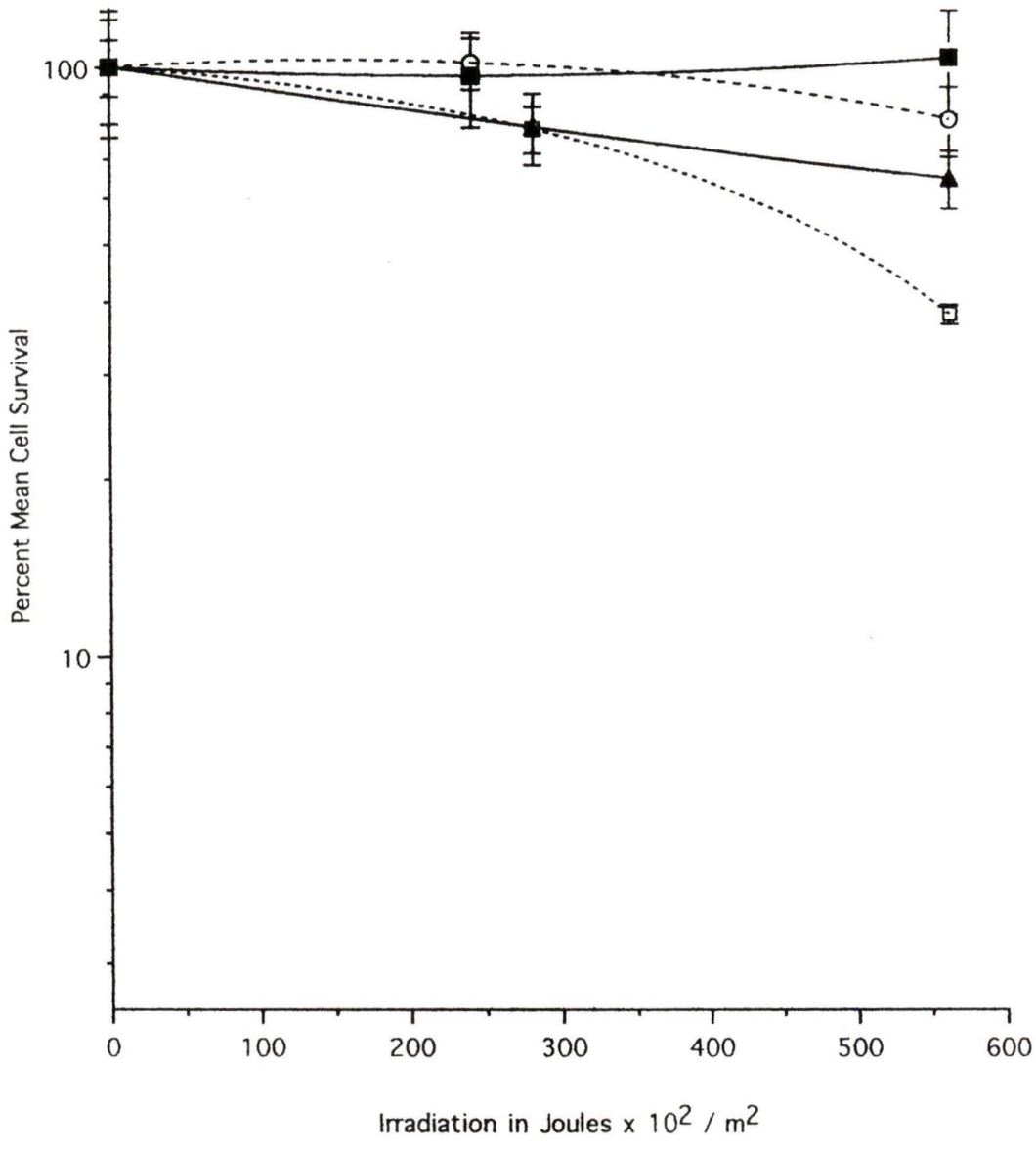
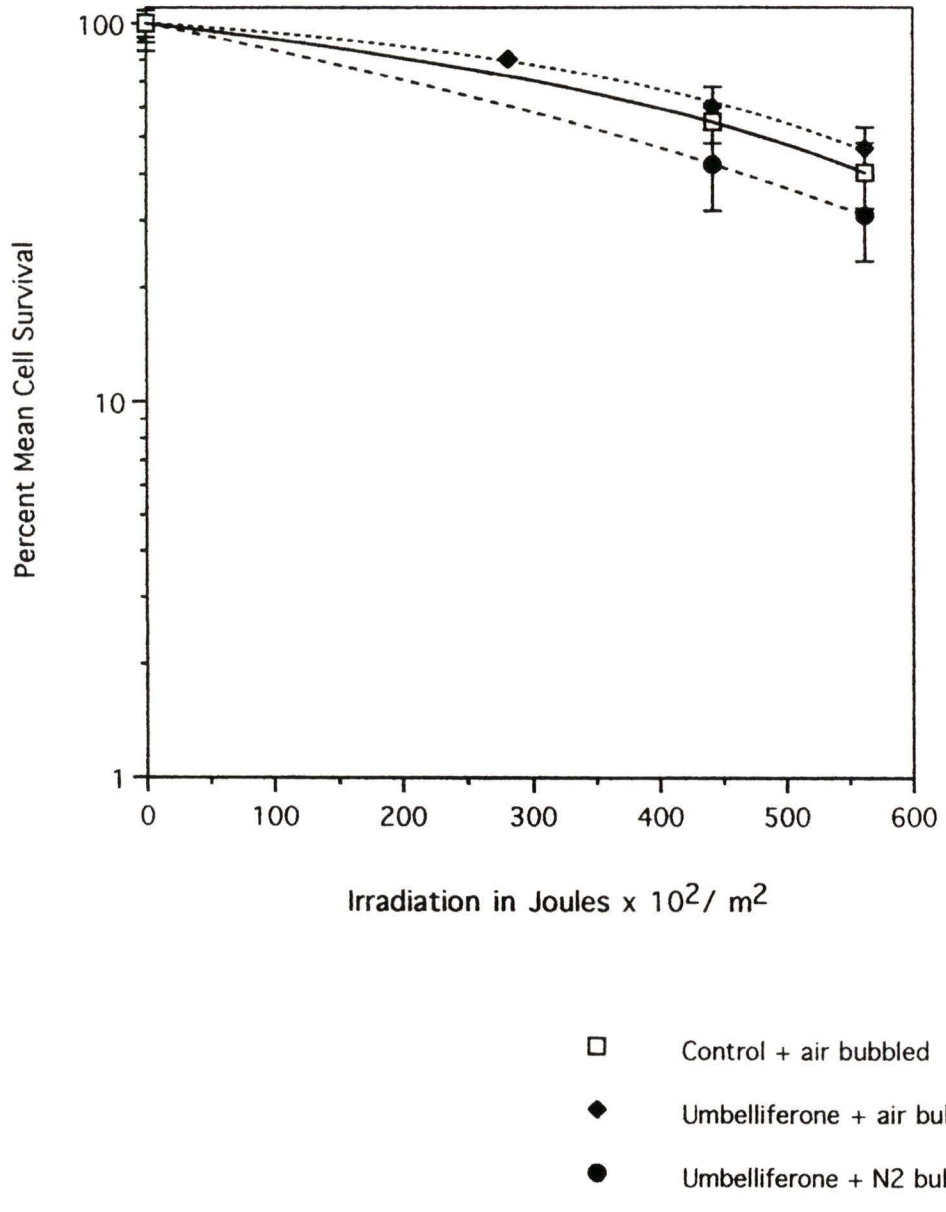

100
10
Percent Mean Cell Survival
0
100
200
300
400
500
600
Irradiation in Joules x 10^2 / m^2
1.5% EtOH control for 40 µg/ml expt.
Umbelliferone @ 40 µg/ml
1.5% EtOH control for 75 µg/ml expt.
Umbelliferone @ 75 µg/ml

Figure 19. Survival of *E. coli B/r WP2 try-* irradiated in phosphate buffer with constant bubbling of air or nitrogen. *E. coli B/r* was grown overnight in nutrient broth to mid-log phase and washed and resuspended in phosphate buffer (0.07 M) to a concentration of approximately 2 x 10^7 cells/ml. Bacteria were irradiated with either a 1.5% ethanol control or 75 μg/ml of umbelliferone. Bacteria were diluted till approximately 2 x 10^3 cells/ml and 100 μl plated on nutrient agar plates and grown overnight. Neither NUV nor umbelliferone had any lethal effects on their own.


100
10
1
Percent Mean Cell Survival
0
100
200
300
400
500
600
Irradiation in Joules x 10²/ m²
Control + air bubbled
Umbelliferone + air bubbled
Umbelliferone + N2 bubbled

Also, umbelliferone was pre-irradiated in phosphate buffer (pH 7.2) with NUV for 2 hrs. (96.48 kJ/m^2) or sunlight for 10 hrs. before irradiation with bacteria to see if that could increase photosensitization. Irradiated solutions turned yellow compared to control solutions. Pre-irradiated either in sun or NUV, umbelliferone was negative for photosensitizing ability. Graphs of results were not shown because of similar results already present in fig. 18 and 19.

A survival curve was also attempted by irradiating wild type *E. coli* with umbelliferone on agar instead of in phosphate buffer solution (see figs. 20, 21, 22 and Table 3). Umbelliferone was incorporated into the base agar at 40 and 75 μg/ml in TNA plates. Results presented for 40 μg/ml are from 1 experiment while 75 μg/ml results are averaged from at least 3 plates from each of 2 experiments. Mean survival for 40 μg/ml with and without NUV was only different by 20.4%. Photographs of plates are presented in fig. 20. Control plates irradiated for 96.48 kJ/m^2 NUV with 1.5% ethanol were negative for photosensitization and 8-MOP at 40 μg/ml produced clear lawns at 48.24 and 96.48 kJ/m^2 as shown by fig. 21 (70 mins. and 2 hrs.).

Only after umbelliferone was increased to a concentration of 75 μg/ml and dosage increased to 96.48 kJ/m^2 of NUV (2 hrs.) was any significant kill produced. Survival decreased to a mean of 36% over 2 experiments ($p = 0.017$ and 0.008). Results from 75 μg/ml are shown in fig. 21 and 22. 8-MOP produced a clear lawn with 56.28 kJ/m^2 (see fig. 20 c and 20f.). Standard deviations of colony numbers were extremely high in umbelliferone plates with highest NUV dose which were not present in others as shown in Table 3. Standard deviations doubled from umbelliferone/dark plates when compared to 2 hrs. NUV at 40 μg/ml and increased four and five fold for experiments with 75 μg/ml.

Colony size also appeared to be smaller in all umbelliferone/NUV plates than control plates with NUV irradiation. Furthermore,

Figure 20. Photographs of plates of *E. coli B/r WP2 try-* survival on agar depicted in fig 21. *E. coli B/r* was grown overnight in nutrient broth to mid-log phase and resuspended in phosphate buffer (0.07 M) and 1:10 dilutions made until concentration was approximately 3-4 x 10^3 cells/ml. 100 µl was spread on TNA agar plates with either a and d)1.5% ethanol or b and e) 40 µg/ml of umbelliferone or c and f) 8-MOP incorporated into it. Plates were either kept in the dark (a,b,c) or irradiated for 70 or 120 mins. (d,e,f) (65.66 or 96.48 kJ/ m^2). At least three plates were used for each irradiation time.

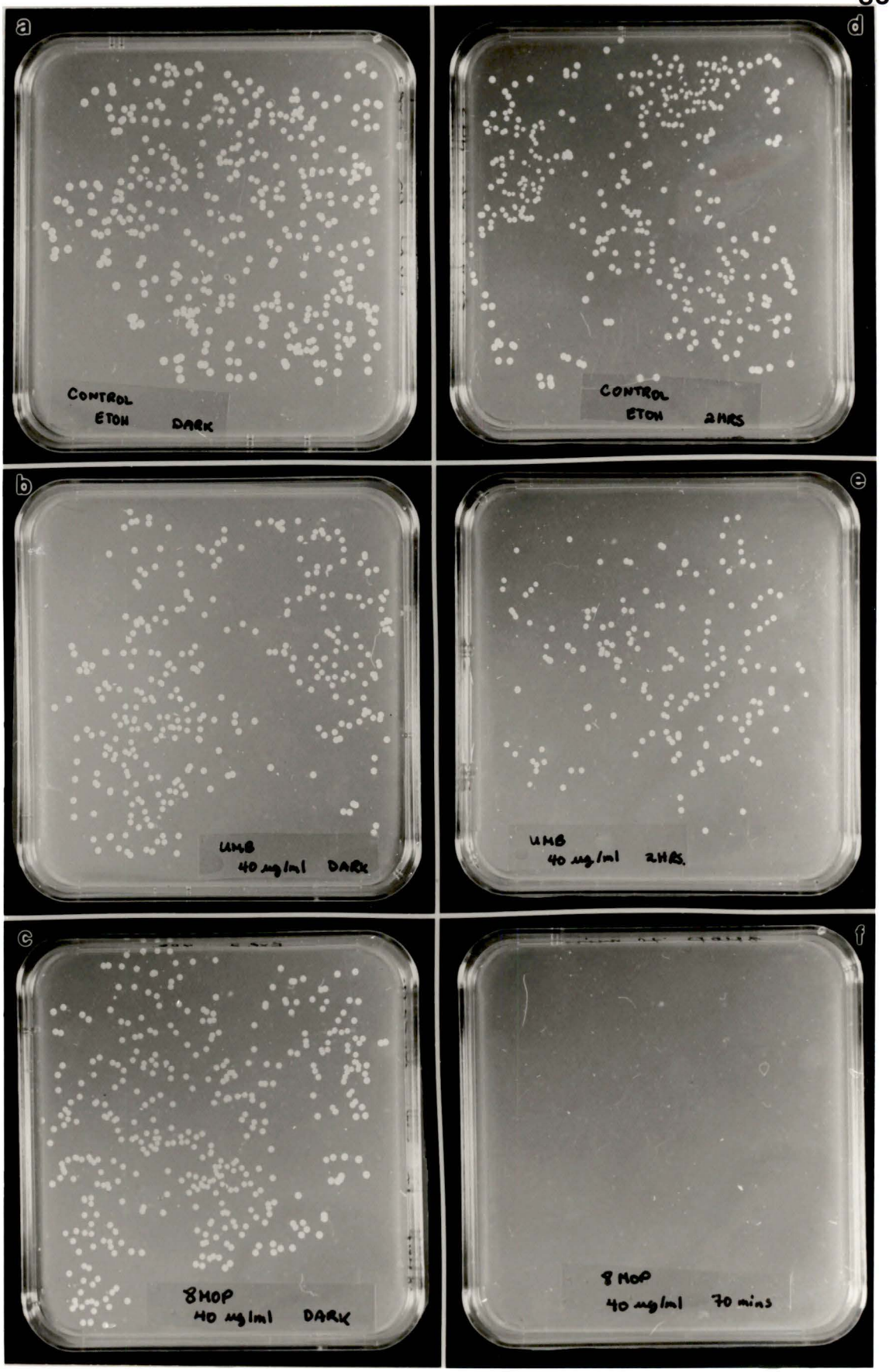

a
CONTROL
ETOH DARK
d
CONTROL
ETOH 2HRS
b
UMB
40 ug/ml DARK
e
UMB
40 ug/ml 2HRS.
c
8MOP
40 ug/ml DARK
f
8 MOP
40 ug/ml 70 mins

Figure 21. *E. coli B/r WP2 try*$^-$ survival on agar. *E. coli B/r* was grown overnight in nutrient broth to mid-log phase and resuspended in phosphate buffer (0.07 M) and 1:10 dilutions made until concentration was approximately 3-4 x 10^3 cells/ml. 100 µl was spread on TNA agar plates with either 1.5% ethanol (control) or 75 µg/ml of umbelliferone or 40 µg/ml 8-MOP incorporated into them. Plates were either kept in the dark or irradiated for 60, 80, or 120 mins. of NUV (48.24, 64.32, or 96.48 kJ/ m^2). At least three plates were used for each irradiation time and results for control and umbelliferone were averaged over at least 2 experiments.

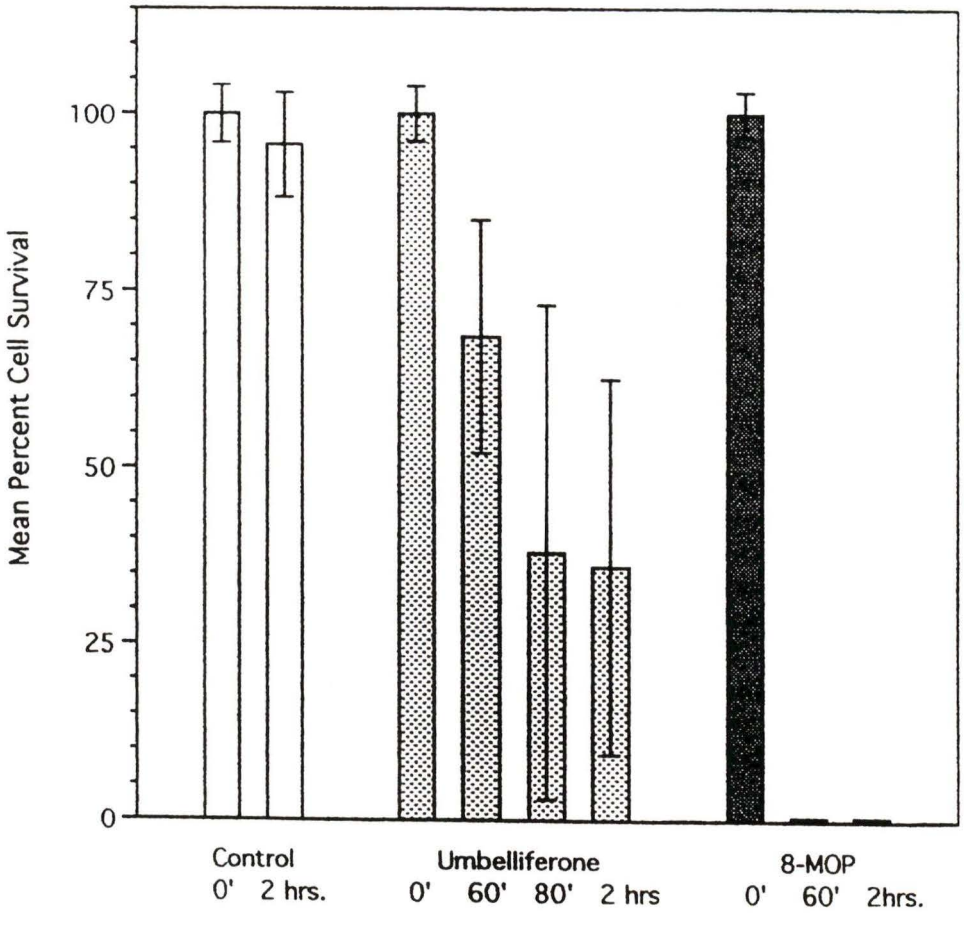

Mean Percent Cell Survival
100
75
50
25
0
Control
0' 2 hrs.
Umbelliferone
0' 60' 80' 2 hrs
8-MOP
0' 60' 2hrs.

Figure 22. *E. coli B/r WP2 try-* survival on agar. *E. coli B/r* was grown overnight in nutrient broth to mid-log phase and resuspended in phosphate buffer (.07 M) and 1:10 dilutions made until concentration was approximately 3-4 x 10^3 cells/ml. 100 µl was spread on TNA agar plates with either a and b)1.5% ethanol or c,d, and e) 75 µg/ml of umbelliferone incorporated into it. Plates were either kept in the dark (a and c) or irradiated x 120 mins. (b,d,e) (96.48 kJ/ m^2). At least three plates were used for each irradiation time.

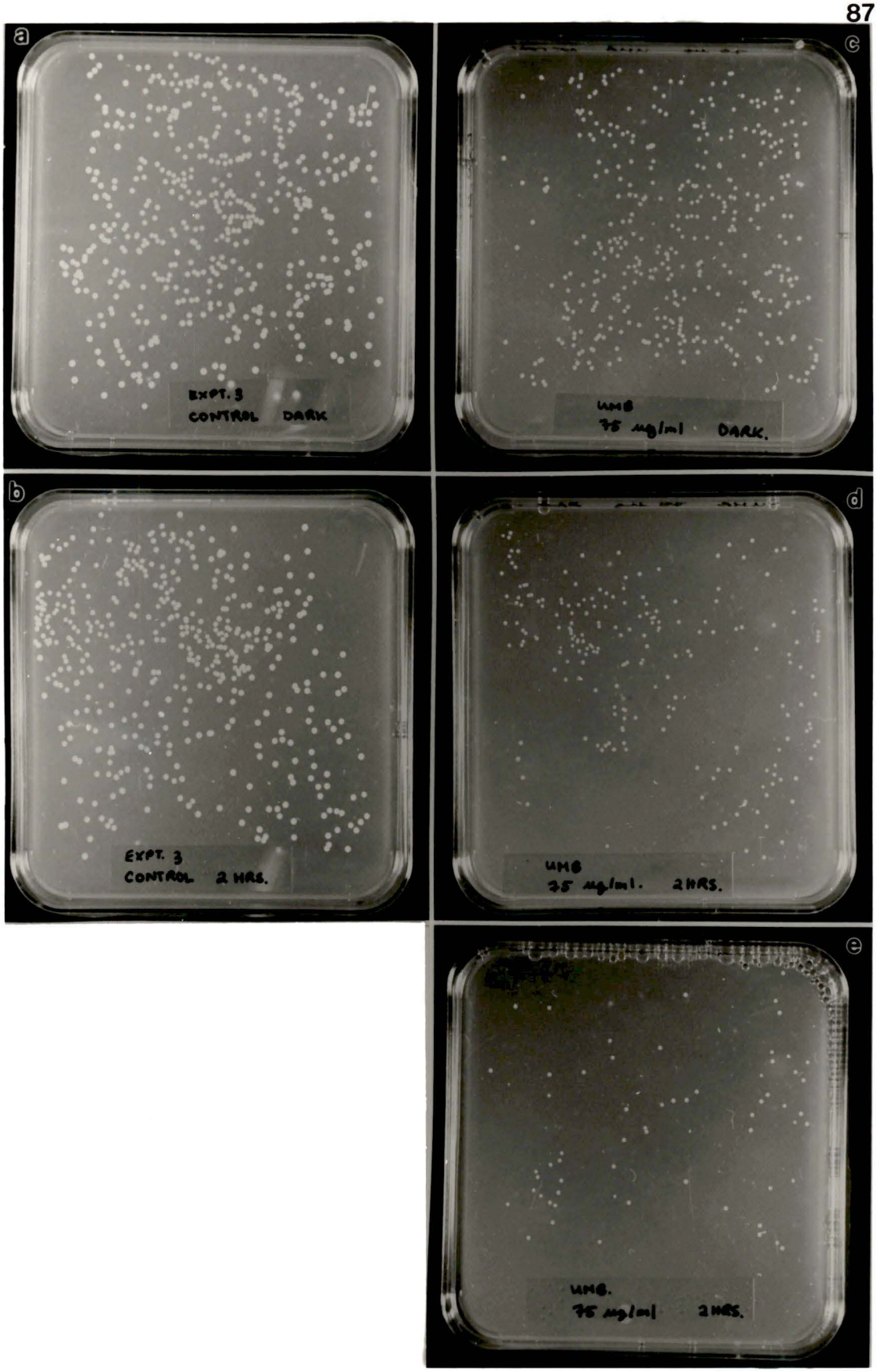

a
EXPT. 3
CONTROL DARK
c
UMB
75 mg/ml DARK.
b
EXPT. 3
CONTROL 2 HRS.
d
UMB
75 mg/ml. 2HRS.
e
UMB.
75 mg/ml 2HRS.

Table 3: Standard Deviation of data from E. coli B/r survival curves from 3 experiments.

Std. Dev. of data from Expt. #	Control Dark	Control 2 hours	8-MOP Dark	Umb. Dark	Umb. Int. Irrad.	Umb. 2 hours
1	-	14.2	-	24	19.4	48.3
2	20.4	33.3	12.9	19.9*	63.5*	80.8*
3	13.2	27.5	-	29.5*	175.2*	161.6*
mean	16.8	25	12.9	24.5	86	96.9

Data given are numbers of colonies

*Umbelliferone 75 ug/ml

All others 40 ug/ml. Control= 1.5% ethanol

Using a Student's t-test:

Standard deviations of control/2 hours versus umbelliferone/dark not significant.

Standard deviations of umbelliferone/dark versus standard deviations of umbelliferone irradiated at 75 ug/ml, $p = 0.035$

colony size on umbelliferone/NUV plates compared to umbelliferone/dark plates also appeared to be smaller. Fig. 20 shows a comparison of colony size using 40 μg/ml of umbelliferone versus the control but these size differences were not statistically significant. Colony size again shows a more marked difference in fig. 22 on 75 μg/ml plates. Not only were dark/umbelliferone treated bacteria colonies smaller than their dark/1.5% ethanol control counterparts, but also irradiated umbelliferone colonies in fig. 22d and 22e were much smaller than dark /umbelliferone colonies shown in fig. 22c. Because of small colony size and difficulties in measuring size, these differences were also not statistically significant.

c) Frameshift mutagenesis of *E. coli ND160 lac⁻ z, thiamine⁻*

As shown by figs. 23 and 24, umbelliferone was tested as a frameshift mutagen against *E. coli ND160 lac⁻,z, thiamine⁻* in the dark at 40, 60, 75, 100, 125 and 175 μg/ml. All plates were done in triplicate and results for 40, 75 and 100 μg/ml were averaged over 3 experiments with remaining data as a result of 1 or 2 experiments. (For details, see figure captions). Background mutation rates varied from 1 per 4.88×10^6 to 1 per 6.67×10^6 cells for all experiments. A background lawn was noted for all concentrations of umbelliferone indicating that all concentrations used were not toxic.

As shown by figs. 23 and 24, umbelliferone was an active frameshift mutagen in this test system. Controls with nothing added to base agar were not significantly different than 1.5% or 2% ethanol. Results were significant to $p < 0.05$ using a Student's t-test for any umbelliferone concentration higher than and including 60 μg/ml when compared to ethanol and had a p value consistently < 0.01 for any concentration of 125 μg/ml or higher. Frameshift revertants increased in a dose dependent manner for all concentrations tested; however, even at 175 μg/ml, umbelliferone did not attain the number of revertants produced by 8-MOP at 40 μg/ml. Umbelliferone induced a mean of 44.3 revertants with a

Figure 23. Frameshift mutagenesis of *E. coli lac-, z, thiamine* - ND 160 in the dark with: control plates (Cont), 1.5% ethanol (EtOH), 40, 75, 125, 175 µg/ml umbelliferone and 40 µg/ml 8-MOP incorporated into the base agar. *E. coli* were grown overnight at 37°C with aeration in nutrient broth and then washed and resuspended in phosphate buffer at approximately 1-2 x 10^8 cells/ml. 100 µl of bacteria was spread onto plates and incubated x 36 hrs. at 37°C. All results were averaged over 3 experiments except for 125 and 175 µg/ml umbelliferone which was the result from one experiment only.

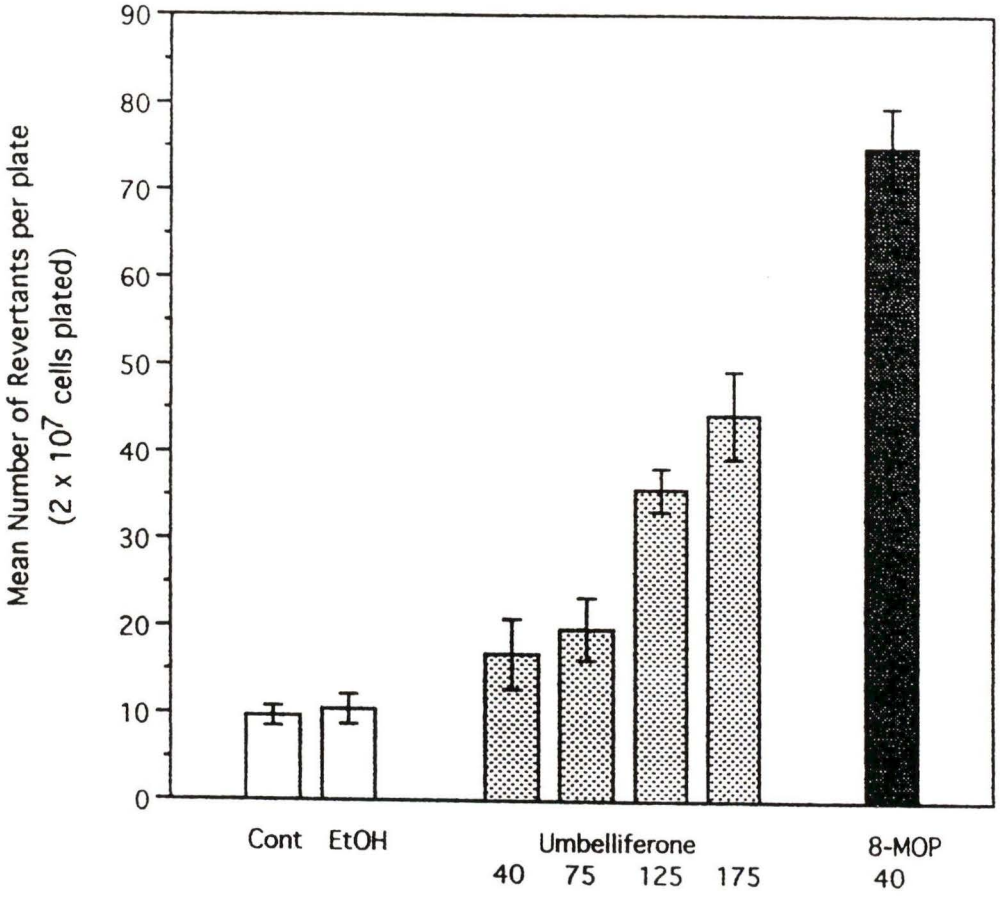
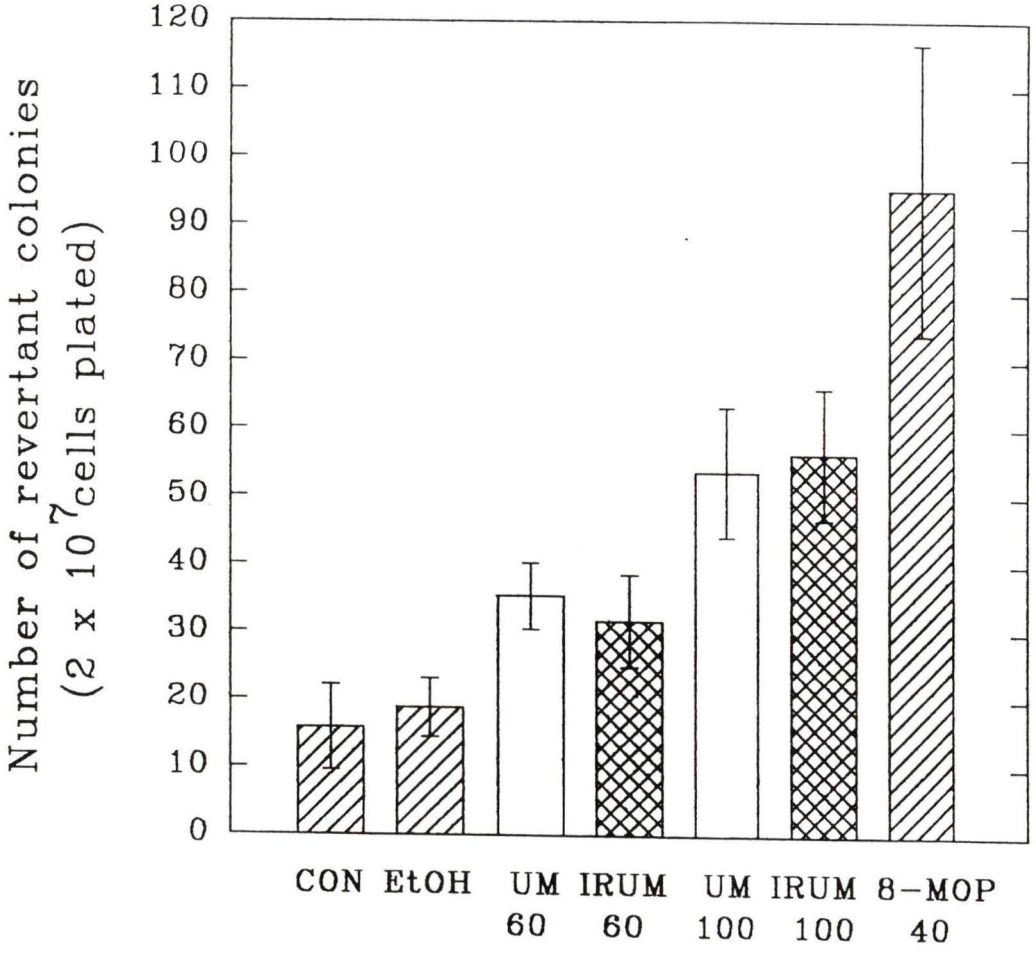

Mean Number of Revertants per plate
(2 x 10^7 cells plated)
90
80
70
60
50
40
30
20
10
0
Cont
EtOH
Umbelliferone
40
75
125
175
8-MOP
40

Figure 24. Frameshift mutagenesis of *E. coli lac-, z, thiamine* - ND 160 in the dark with: control plates (Cont), 2% ethanol (EtOH), 60 and 100 µg/ml standard umbelliferone (UM) or irradiated umbelliferone (IRUM) and 40 µg/ml 8-MOP incorporated into the base agar. *E. coli* were grown overnight at 37°C with aeration in nutrient broth and then washed and resuspended in phosphate buffer at approximately 1-2 x 10^8 cells/ml. 100 µl of bacteria was spread onto plates and incubated x 36 hrs. at 37°C. Irradiated results are from one experiment only while standard umbelliferone 60 and 100 µg/ml are from 1 and 3 experiments respectively.


Number of revertant colonies
(2 x 10^7 cells plated)
120
110
100
90
80
70
60
50
40
30
20
10
0
CON
EtOH
UM 60
IRUM 60
UM 100
IRUM 100
8-MOP 40

standard deviation of 5.8 while 8-MOP induced a mean of 75 revertants with a standard deviation of 4.5 ($p < 0.0001$ when compared to control). Photographs of plates are given in fig. 25.

As biological results had been so varied thus far and the active photobiological moiety was still yet to be determined between umbelliferone and its photoproducts, irradiated umbelliferone was also tested for frameshift ability. Plates of 60 and 100 µg/ml of umbelliferone were poured and half were pre-irradiated under NUV for 96.48 kJ/m^2 (2 hrs.) to form photoproducts in the agar. The experiment was then carried out with bacteria in the dark as per materials and methods. As shown by fig. 24, no significance could be found between the number of frameshift revertants produced by irradiated and non-irradiated umbelliferone. However, both 60 and 100 µg/ml were again significant ($p < 0.05$) when compared to control.

Because frameshift mutagenesis was negative for the following Ames test, this experiment was also repeated in an overlay method. As umbelliferone is very water soluble and was only added to the 2 ml of overlay in the Ames test, umbelliferone was tested in a 2 ml soft agar overlay method at 100, 200, and 250 µg/ml of overlay. As shown by fig. 26, only until 250 µg/ml was used was any significant results obtained ($p < 0.005$ on Student's t-test). 20 ml of base agar were used in each plate under overlay and it was observed after 2 days incubation that the characteristic umbelliferone fluorescence under UV was diffused uniformly throughout the overlay and the base agar. A background lawn was also noted on the overlay indicating that the high umbelliferone concentration used was not toxic.

d) Frameshift mutagenesis via Ames *Salmonella typhimurium TA98* test

Frameshift mutagenesis was also tested on *Salmonella typhimurium TA98* strain in the overlay method described by Maron and Ames (1983). The control, umbelliferone (at 50 and 150 µg/ml

Figure 25. Photographs of plates of frameshift mutagenesis of *E. coli lac-, z, thiamine-* ND 160 in the dark with either a) 2% ethanol, b) 60 µg/ml umbelliferone, d) 100 µg/ml umbelliferone, or c) 40 µg/ml 8-MOP incorporated into the base agar. *E. coli* were grown overnight at 37°C with aeration in nutrient broth and then washed and resuspended in phosphate buffer at approximately 1-2 x 10^8 cells/ml. 100 µl of bacteria was spread onto plates and incubated x 36 hrs. at 37°C.

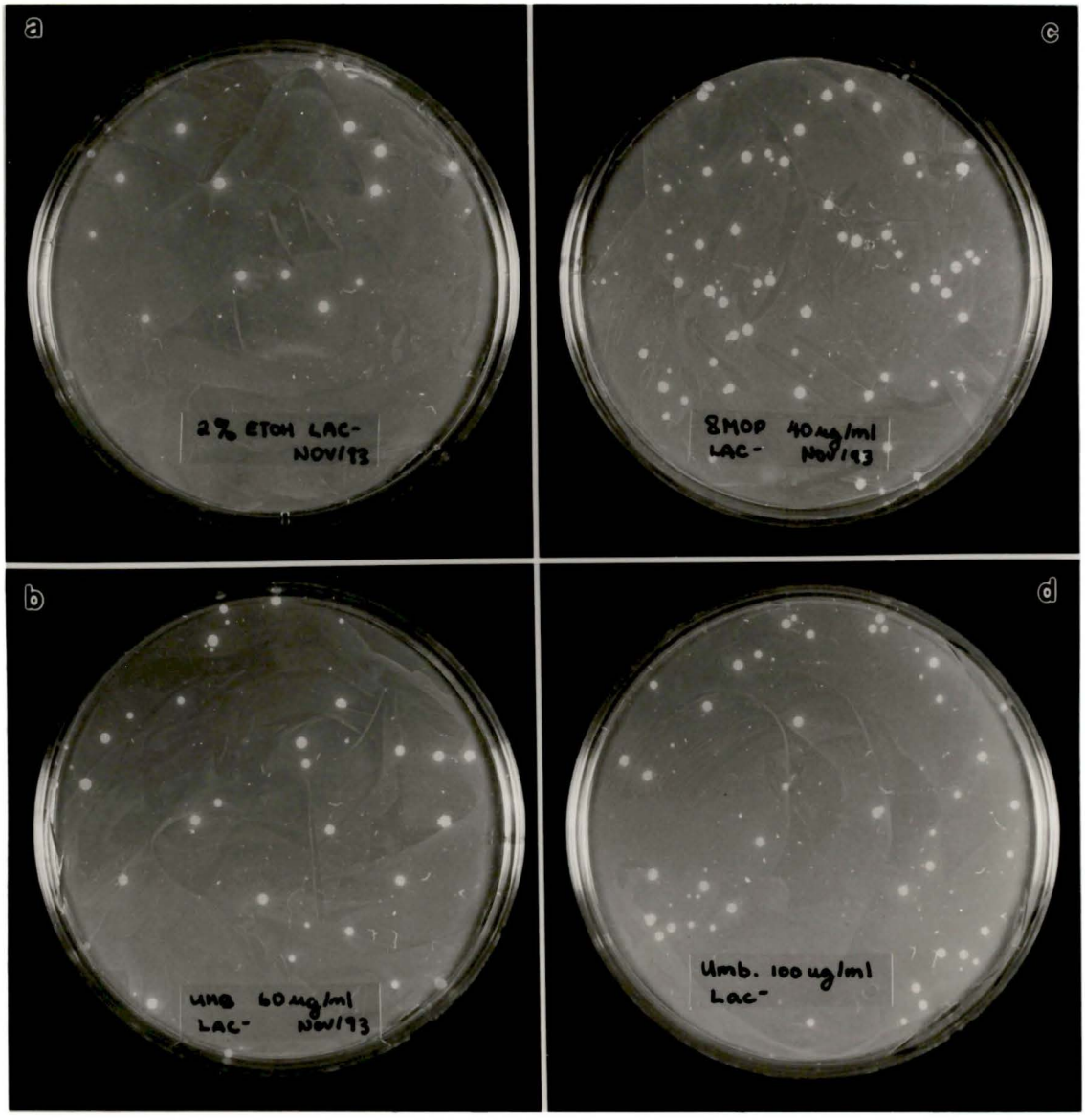
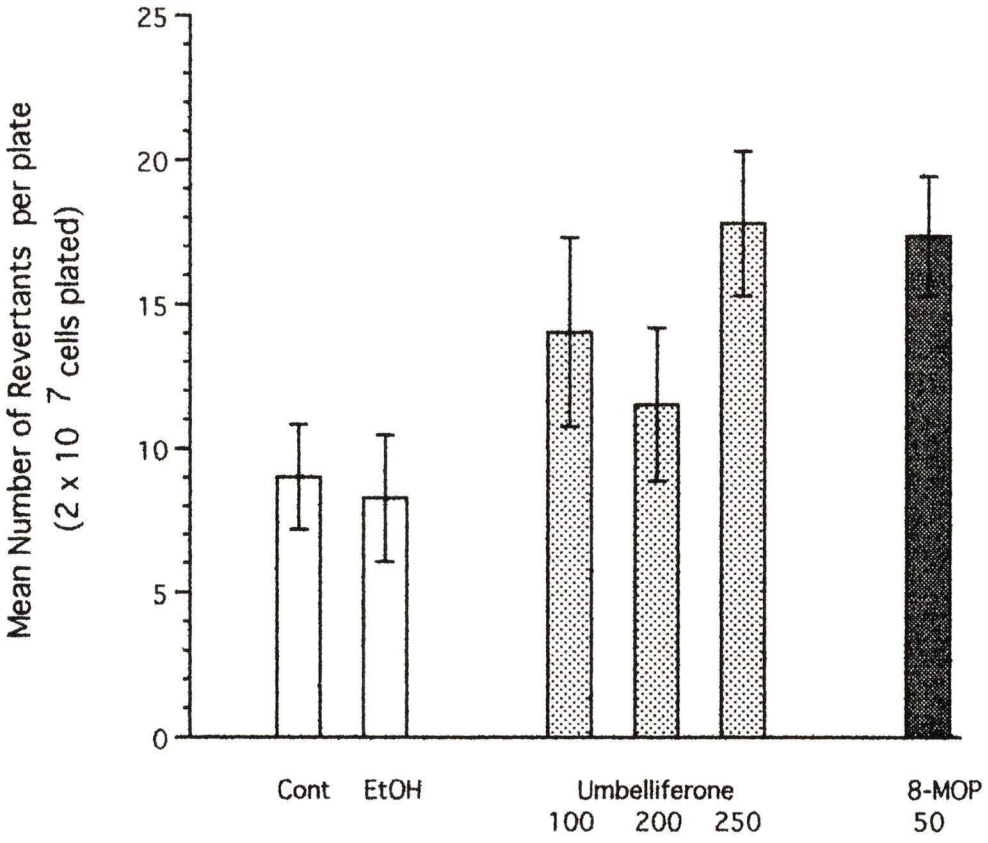

a
2% ETOH LAC-
NOV/93
c
8MOP 40ug/ml
LAC- NOV/93
b
UMB 60ug/ml
LAC- NOV/93
d
Umb. 100ug/ml
Lac-

Figure 26. Frameshift mutagenesis of *E. coli lac-, z, thiamine-* ND 160 in the dark in an overlay method. Control agar overlay (Cont) or with 1.5% ethanol, 100, 200, or 250 µg/ml umbelliferone or 50 µg/ml 8-MOP incorporated into the 2 mls of overlay was used. *E. coli* were grown overnight at 37°C with aeration in nutrient broth and then washed and resuspended in phosphate buffer at approximately 1-2 x 10^8 cells/ml. 100 µl of bacteria was added to overlay and poured onto plates and incubated for 36 hrs. at 37°C.


Mean Number of Revertants per plate
(2 x 10 7 cells plated)
25
20
15
10
5
0
Cont
EtOH
Umbelliferone
100
200
250
8-MOP
50

in 2 ml of overlay) and 1 μg of benzo(a)pyrene all proved negative without S9 activation as shown by fig. 27. Background mutation rate was slightly elevated at 1 per 2.5 x 10^6 cells. Controls and umbelliferone plates with S9 activation did not have any significant difference over those without S9 and umbelliferone did not increase revertants significantly when compared to control. Benzo(a)pyrene when combined with S9 microsome mix, as the positive control, increased revertants more than 5 times the background rate ($p < 0.0001$).

e) Lethal photosensitization of Chinese hamster ovary cells

Umbelliferone was tested against CHO cells for photosensitizing ability at 40 and 160 μg/ml (see fig. 28 and 29). Results for 40 μg/ml were averaged over 2 experiments and all flasks were seeded in triplicate. Data for 160 μg/ml was collected from 1 experiment only. Cloning efficiency varied from 40 to 54% for each experiment. As shown by fig. 28, umbelliferone did not produce any significant photosensitization at 40 μg/ml. 160 μg/ml of umbelliferone also had no effect but results are not shown as they were essentially identical to those at 40 μg/ml. 20.1 kJ of NUV irradiation was used per square meter (25 mins.). Control survival decreased to a mean of 25.6% for the three experiments at 20.1 kJ., and thus increasing irradiation dosages were not done. 8-MOP (40 μg/ml), as the positive control, produced 93% kill after only 804 Joules/m^2 ($p< 0.01$ using Student's t-test). As can be shown by photographs of flasks in fig. 29, no difference can be seen between control and umbelliferone flasks.

f) Inactivation of ampicillin resistance gene in pTZ18R

Although the difference in susceptibility to umbelliferone between wild type and DNA-repair deficient *E. coli* in the photobioassay and frameshift mutagenesis of *E. coli lac-* strongly suggested that DNA was the target of umbelliferone's activity, irradiation of DNA alone with umbelliferone and NUV was done to

Figure 27. Frameshift mutagenesis of *Salmonella typhimurium* TA98 in the Ames test with and without S9 microsomal activation. Either phosphate buffer as the negative control, 100 or 300 µg of umbelliferone or 1 µg of benzo(a)pyrene was incorporated into the 2 mls of overlay. Bacteria were grown overnight at 37°C with aeration to 1-2 x 10^9 cells/ml and 100 µl added to each overlay tube and poured onto Ames minimal medium plates. Plates were then incubated for 2 days at 37°C.

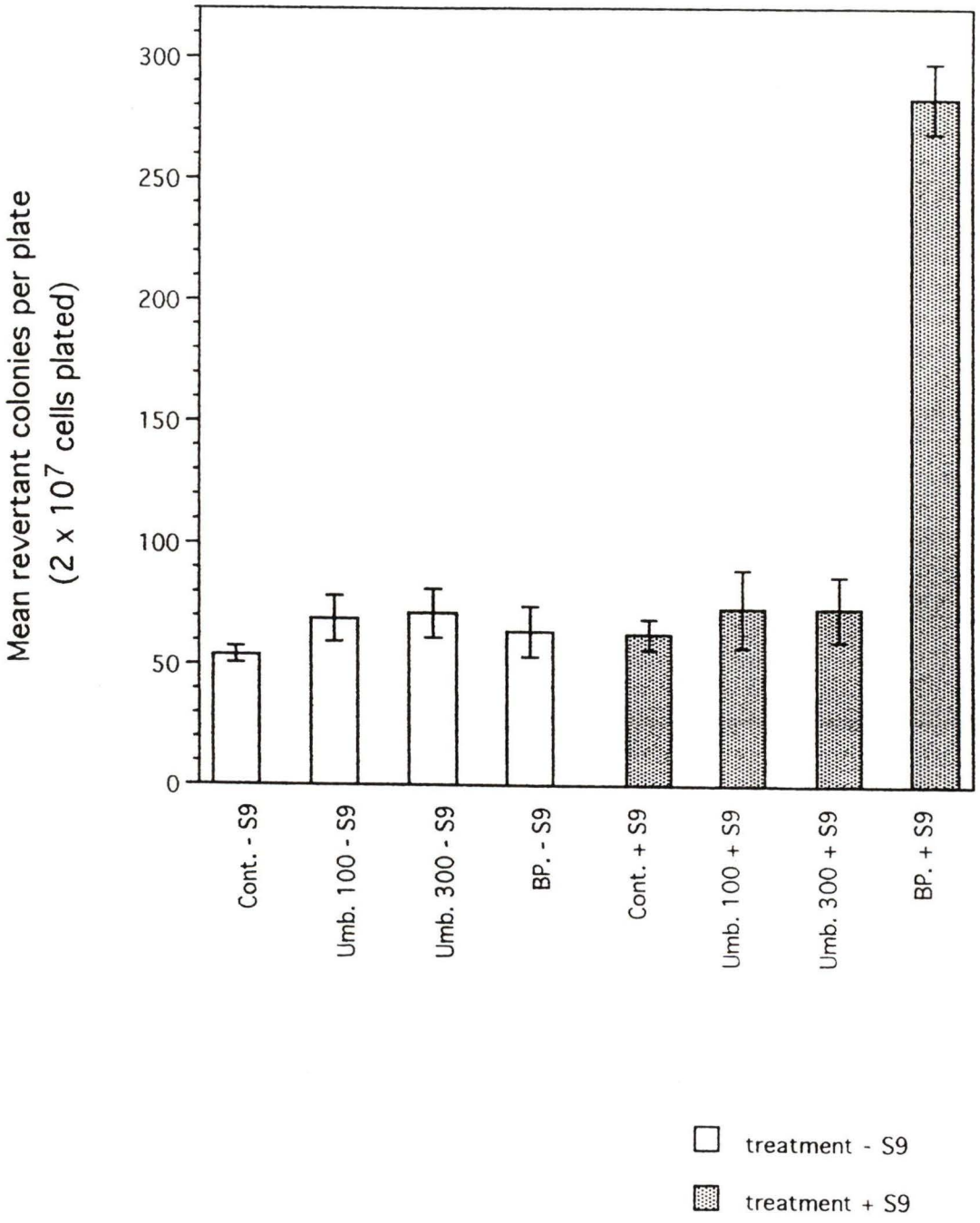

Mean revertant colonies per plate
(2 x 10^7 cells plated)
300
250
200
150
100
50
0
Cont. - S9
Umb. 100 - S9
Umb. 300 - S9
BP. - S9
Cont. + S9
Umb. 100 + S9
Umb. 300 + S9
BP. + S9
treatment - S9
treatment + S9

Figure 28. Survival of Chinese hamster ovary cells after treatment with NUV irradiation (320-380 nm; peak 350 nm) in the presence of either 1.5% ethanol or 40 μg/ml of umbelliferone or 8-MOP. Cells were allowed to attach and irradiated in McCoy's 5a medium minus fetal calf serum with above compounds. Survival was measured through formation of colonies from a 10 day incubation period after irradiation. Neither NUV nor umbelliferone by itself had any toxic effects.

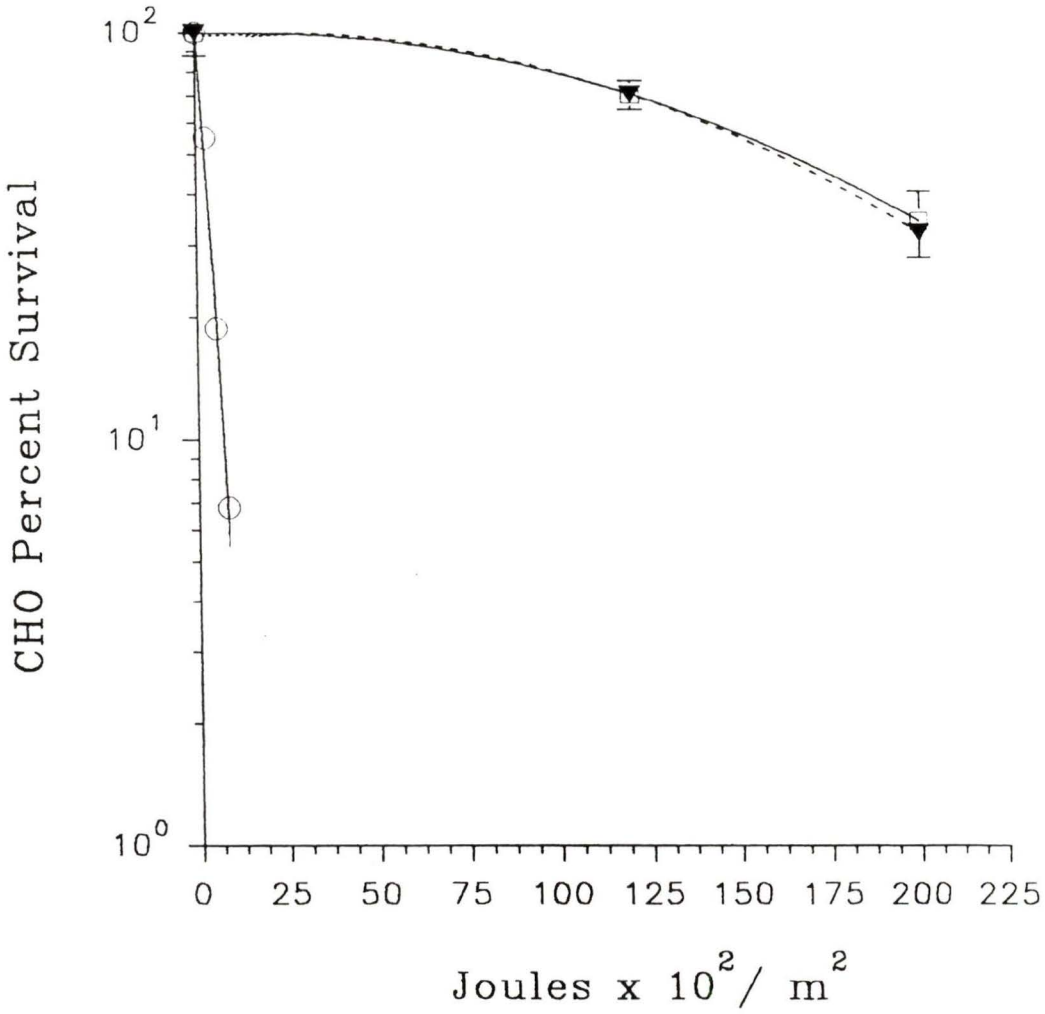

CHO Percent Survival
10^2
10^1
10^0
0 25 50 75 100 125 150 175 200 225
Joules x 10^2/ m^2
umbelliferone
8-MOP
control
Error bars display
standard deviation

Figure 29. Photographs of Chinese hamster ovary tissue culture flasks used for survival data depicted in fig. 28. Cells were treated with NUV irradiation (320-380 nm; peak 350 nm) in the presence of either a and c) 1.5% ethanol or b and d) 40 μg/ml umbelliferone. a and b are dark controls while c and d have been exposed to 20.1 kJ/ m^2 NUV. Survival was measured as formation of colonies from a 10 day incubation period after irradiation. Neither NUV nor umbelliferone by itself had any toxic effects.


a
c
b
d

confirm that umbelliferone could photoreact with DNA. A series of 4 experiments was done to determine a baseline transformation efficiency curve for the plasmid pTZ18R DNA and Epicurian Coli SURE Cells ™ for lot #1 of cells and results are given in fig. 30. This curve was reconfirmed by 2 experiments for lot #2 of SURE Cells ™ as shown by fig. 31. These experiments were done to determine the number of ampicillin resistant transformants a known amount of plasmid DNA would produce and to ensure that a 90% decrease in transcribable DNA would give a similar decrease in number of transformants. As can be shown by both figs., 4.0 ng plasmid DNA was found to be in the linear portion of the transformation efficiency curve.

pTZ18R DNA was used in various amounts between 0.04 and 4.40 ng to determine a transformation curve for lot # 1 of SURE ™ cells. Transformation efficiency varied greatly between the two lot numbers of cells used for these experiments but only slightly between experiments of the same lot number. As shown by Table 4, mean transformation efficiency for lot #1 for experiments 2-5 was 3.48×10^6 colonies/μg of DNA with an S.D. of 1.05×10^6 (30%) (n=17). In comparison, table 5 shows that mean transformation efficiency for lot #2 for experiments 6 -10 was 7.40×10^5 colonies/μg of DNA with an S.D. of 2.67×10^5 (36%) (n=16). As shown by this data, transformation between lot#'s 1 and 2 of cells varied 10 fold: 10^6 colonies/μg of DNA vs. 10^5 colonies/μg. However, the slope of the line for transformants versus amount of DNA used is similar as shown by data from experiment 6 and 7 in fig. 31 compared to fig. 30 even though actual numbers are less.

This comparison was illustrated again in calculating percentage of cells transformed by different amounts of DNA (see Table 4). For lot#1 and experiments 4 and 5, 4.0 ng of DNA transformed an average of 0.037% of cells plated (S.D. 0.0060%, n=7) with percentage of cells transformed decreasing with amount of DNA. Using data from experiments 2,3, and 5, 0.8 ng-1.0 ng transformed an average of 0.016% of cells with a S.D. of 0.0078%. In comparison, 4.0 ng of DNA

Figure 30. Epicurian Coli SURE ™ cell transformation as a function of amount of pTZ18R DNA used. Results depicted are from 4 experiments using Lot # 1 of cells.
Transformation procedure was done through an ice and heat shock method and colonies were grown up overnight.

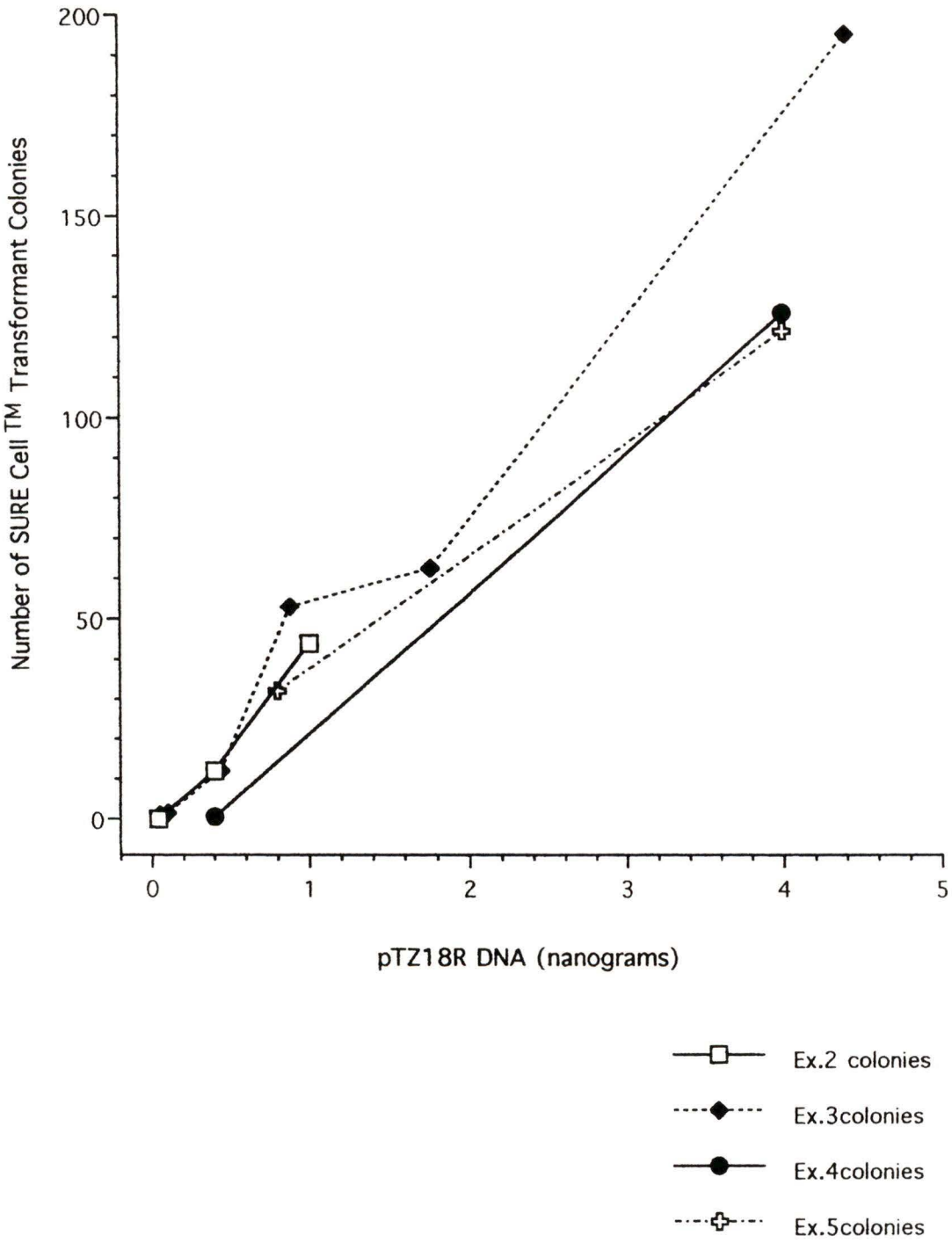

200
150
100
50
0
Number of SURE Cell TM Transformant Colonies
0
1
2
3
4
5
pTZ18R DNA (nanograms)
Ex.2 colonies
Ex.3colonies
Ex.4colonies
Ex.5colonies

Figure 31. Epicurian Coli SURE ™ cell transformation as a function of amount of pTZ18R DNA used. Results depicted are from 2 experiments using Lot # 2 of cells. Transformation procedure was done through an ice and heat shock method and colonies were grown up overnight.

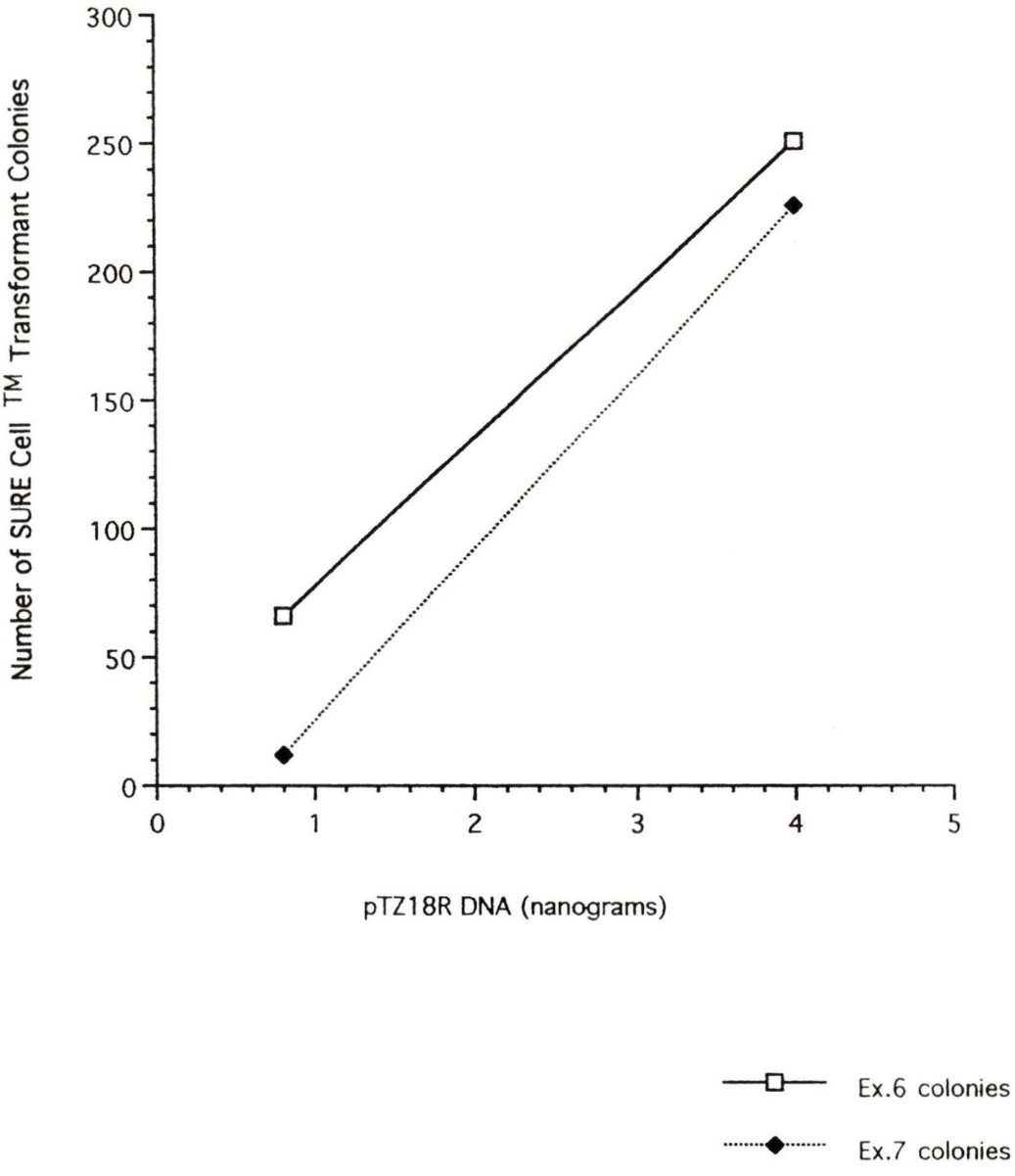

Number of SURE Cell TM Transformant Colonies
300
250
200
150
100
50
0
0
1
2
3
4
5
pTZ18R DNA (nanograms)
Ex.6 colonies
Ex.7 colonies

Table 4 Transforming efficiency and percentage of cells transformed for pTZ18R and Lot #1 SURE Cells™

Expt. #	Quantity of pTZ18R DNA	Mean viability x 1E+04 cells	Std. Dev. x1E+04	p value	Transforming efficiency /µg DNA	% cells trans-formed
2	1.000 ng A				4.90E+06	0.0160
	1.000 ng B				3.85E+06	0.0130
	0.400 ng				5.30E+06	0.0100
	0.004 ng	333.9	77.5		0.00E+00	0.0000
3	4.400 ng				4.42E+06	0.0970
	1.760 ng				3.55E+06	0.0300
	0.880 ng				5.34E+06	0.0270
	0.440 ng				3.03E+06	0.0067
	0.088 ng				2.33E+06	0.0010
	0.044 ng	194.8	60.6		2.95E+06	0.0007
4	4.0 ng A	263.0	18.4	n.s.	3.14E+06	0.0465
	4.0 ng B				2.29E+06	0.0340
	4.0 ng H20/IR				2.25E+06	0.0330
	4.0 ng DMSO				2.40E+06	0.0360
	4.0 ng DMSO/IR	260.2	46.0	n.s.	3.00E+06	0.0440
AVG	4.0 ng (Ex.4)				2.62E+06	0.0390
5	0.8 ng				4.03E+06	0.0087
	4.0 ng	360.0	18.5	n.s.	2.90E+06	0.0320
	4.0 ng 8-MOP	377.0	21.0	n.s.	3.03E+06	0.0320

AVG Trans. efficiency for Expt. 2,3,4, & 5 (n = 17) **3.48E+06**
(S.D. = 1.05E+06)

AVG % cells transformed for 4.0 ng Expt. 4 &5 **0.037%**
(S.D.= .006%)

AVG % cells transformed for 0.8-1.0 ng for Expt. 2,3 & 5 **0.016%**
(S.D.=.008%)

n.s.: not significant in student's t-test; DMSO (0.1% v/v)
IR: NUV irradiated x 8.04 kJ/ square meter; 8-MOP (5 µg/ml)

Table 5 Transforming efficiency and percentage of cells transformed for pTZ18R and Lot #2 Sure Cells™

Expt. #	Treatment	Mean viability x 1E+05 cells	Std. Dev. x 1E+05	p value	Transforming efficiency /µg DNA	% cells transformed
6	control 0'	52.1	2.9	n.s.	6.25E+05	0.0045
	8-MOP 10'	57.8	4.8	n.s.	1.15E+05	0.0008 **
7	control 0'	71.3	-	-	5.26E+05	0.0030
	COR 10'	76.2	-	-	0.00E+00	0.0000 **
8	control 30'	70.2	10.3	n.s.	8.77E+05	0.0054
	UMB 30'	81.8	7.8	n.s.	1.18E+06	0.0064
9	UMB 0'*	103.7	18.5	n.s.	1.84E+06	0.0074
	UMB 120'*	96.0	9.7	n.s.	1.00E+04	0.0011 **
10	control 120'	91.9	4.7	n.s.	7.70E+05	0.0034
	UMB 120'*	77.8	14.4	n.s.	7.25E+04	0.0003 **

AVG Trans. efficiency for Expt.'s 6 -10 (n=16) **7.40E+05**
(S.D. = 2.67E+05)

AVG % cells transformed for 4.0 ng Control DNA **0.0041%**
(S.D. = 0.0001%)

* Umbelliferone concentration 15 µg/ml. All others 5 µg/ml.
Control= 0.1% DMSO.

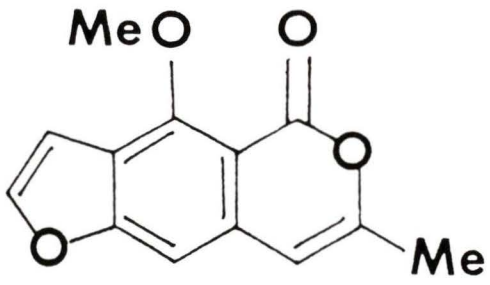
**Low transforming efficiency and % cells transformed due to decrease of transformable DNA from treatment.
Experiment 7 shows no standard deviation/p value due to only 2 plates counted for data.
n.s.: not significant in student's t-test

for lot #2, as shown by Table 5, transformed an average of 0.0045% of cells plated with a S.D. of 0.0015% (n=12) (all data not shown). This was again about 10 fold less than lot#1. 0.8 ng of DNA in lot #2 transformed an average of 0.00068%. (n=2). Despite these differences, the percentage of cells transformed decreased when the amount of DNA decreased from 4.0 ng to 0.8 ng for both lots of SURE ™ cells.

Not only was the transformation efficiency for cells within the same lot number confirmed to be fairly stable, the numbers of viable cells used for each transformation were also checked to ensure that they were not significantly different from one another. 100 µl of a $1/10^4$ and $1/10^5$ dilution of 2 out of the 6 transformation broth cultures in each experiment were plated on nutrient agar. No significant difference was ever found within any experiment between numbers of cells used for different transformations as shown by experiment 4 and 5 in Table 4 and Table 5. For example, in experiment 10, a mean of 91.9 cells with an S.D. of 4.7 grew on NA plates from a $1/10^5$ dilution from the culture transformed with control pTZ18R DNA treated with 2 hrs NUV. Similarly, 77.8 cells with an S.D. of 14.4 grew from a $1/10^5$ dilution from a culture transformed with pTZ18R DNA treated with umbelliferone 15 µg/ml/ 2 hrs NUV. Thus, any difference in numbers of transformants could not be attributed to differences in numbers of viable cells used for each transformation or in differences in transformation efficiency.

With above criteria established, control DNA at 4.0 and 0.8 ng along with 4.0 ng DNA treated with either 8-MOP (5 µg/ml) or coriandrin (5 µg/ml) and NUV irradiation were used to compare transformation ability. Coriandrin, a furanocoumarin, is depicted in fig. 32 and has been shown to form monoadducts only when irradiated with DNA (Ashwood-Smith et al., 1989). Six different transformations could be done within one experiment. Results for 8-MOP are collected from one experiment while results depicted for control and coriandrin are averaged from 3 and 2 separate

Figure 32. Chemical structure of the furanocoumarin, coriandrin (4-methoxy-7-methyl-5H-furo[2,3-*g*] [2]benzopyran-5-one).


MeO
O
O
O
Me

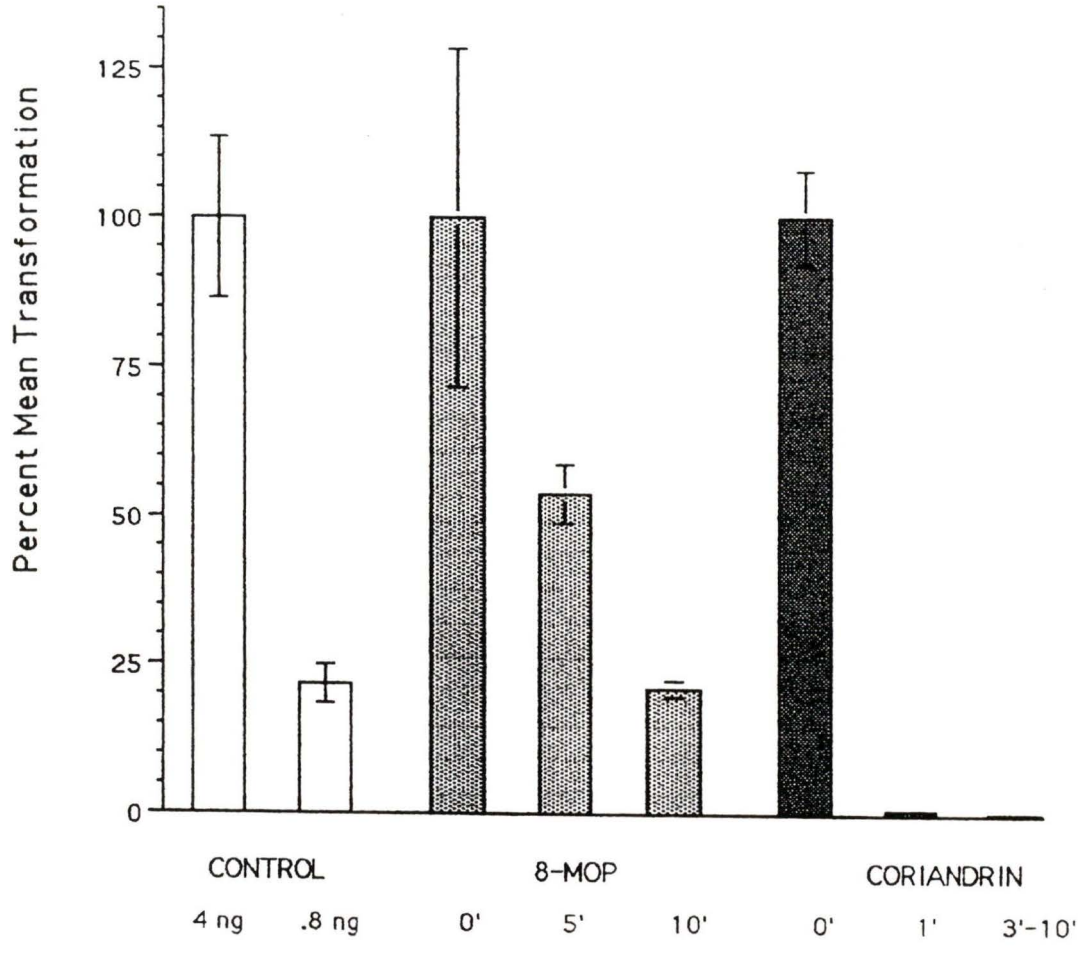
experiments respectively. Results were averaged from experiments and are presented in fig. 33.

As shown by fig. 33, using 100% as number of ampicillin transformants obtained from 4.0 ng of control DNA, mean percent transformation dropped to 21.7% for 0.8 ng. Irradiation with NUV for 10 mins. had no effect at all on transformation ability of control DNA. Using 100% as number of transformants from 4.0 ng DNA with test compound/- NUV, numbers of transformants for 8-MOP/NUV treated DNA dropped from 100% at 0 min. NUV to 53.7% at 5 mins. and 21.0% at 10 mins. (4.02 kJ and 8.04 kJ/m^2) ($p = 0.024$ for 4.02 kJ and $p = 0.009$ for 8.04 kJ using a Student's t-test).

For this experiment, duplicate transformations labelled 5'A and 5'B were done for 8-MOP and 5 mins. NUV. An average was taken of both vials and results shown in fig. 33. Vial A had an average of 88.3 transformants (S.D. = 12.5) while vial B had an average of 129.7 transformants (S.D. = 9.07). The differences between vials was significant ($p = 0.01$) with vial A having 68% the number of transformants as vial B. Levels of significance were $p = 0.24$ between data for 8-MOP 0' and 5'A NUV and $p = 0.076$ for 5'B. Levels of significance were $p = .007$ for comparison of 5'A and 10' and $p < 0.0005$ for 5'B and 10'. There was no significant difference between 4.0 ng control DNA and 4.0 ng 8-MOP/0' NUV.

Mean percentage of transformants for coriandrin/ NUV treated DNA dropped from 100% at 0 mins. NUV to 0.8% at 1 min. (804 Joules) and 0% at any irradiation time between 3 and 10 mins. of irradiation as shown by fig. 33 (2.41 kJ to 8.04 kJ/m^2) (all p values < 0.0005). No significant difference was found between numbers of transformants for control 4.0 ng +/- NUV and therefore are graphed together. No difference was also found between numbers of transformants for control 4.0 ng and 4.0 ng Coriandrin without NUV irradiation. Numbers of transformants obtained from 4.0 ng and 0.8 ng control DNA were also significant at $p = 0.001$.

Figure 33. Transformation of Epicurian Coli SURE ™ cells by pTZ18R treated with either 0.1% DMSO (Control), 5 µg/ml 8-MOP or coriandrin with or without NUV light. 4.0 ng of plasmid DNA was used for each transformation procedure unless otherwise indicated. Control and coriandrin results were averaged over 2 experiments while 8MOP results were from 1 experiment only. NUV irradiation was performed on each of the three test treatments of plasmid for 0, 1, 3, 5, or 10 mins. as indicated in the presence of compound, 0.1% DMSO and millipore water only (0, 0.804, 2.412, 4.02, or 8.04 kJ/ m^2) and then appropriate aliquots added to cells in described transformation procedure. Transformations were done with Lot #2 cells.

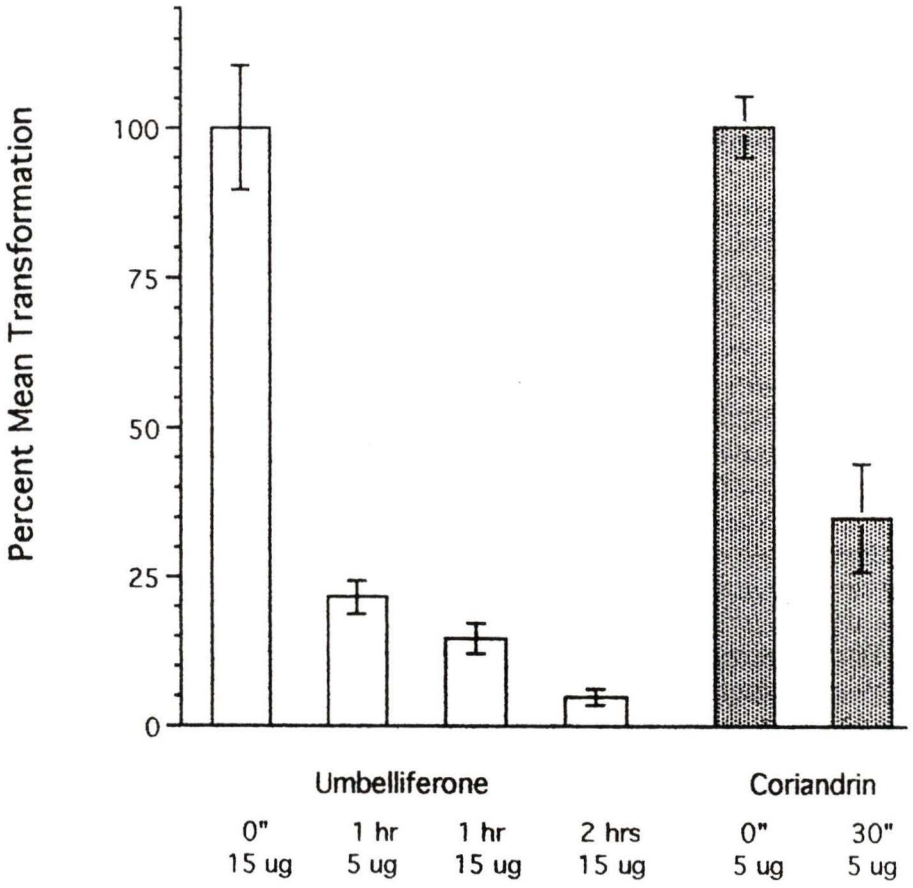


Standard Deviation Bars shown

With experiment protocol established, pTZ18R DNA was treated with umbelliferone at 5 μg/ml and 15 μg/ml and NUV irradiation. Umbelliferone at 5 μg/ml had no effect on transforming ability after 0 and 30 mins. of irradiation (12.06 and 24.12 kJ/m^2) ($p = 0.54$). (Data not shown). With longer irradiation times and higher concentrations of umbelliferone, an effect on transforming ability was found as depicted by fig. 34. A clear dose response relationship is depicted in fig. 34 with a decrease in DNA transformation ability with either an increase in concentration of umbelliferone or NUV dose. pTZ18R treated with umbelliferone dropped from 100% of transformants to 21.6% at 1 hr NUV (48.24 kJ/m^2) using 5 μg/ml and then to 14.7% at 1 hr at 15 μg/ml. Mean percentage of transformants dropped to 4.9% at 2 hrs (96.48 kJ/m^2) at 15 μg/ml of umbelliferone. All results were statistically significant at $p < 0.0005$. Coriandrin was tested alongside at 0 and 30 seconds NUV at 5 μg/ml for a positive control and dropped from 100% to 34.9% ($p < 0.0005$). Because of a limitation of testing only 6 samples within one complete experiment, no negative control was used alongside in this transformation.

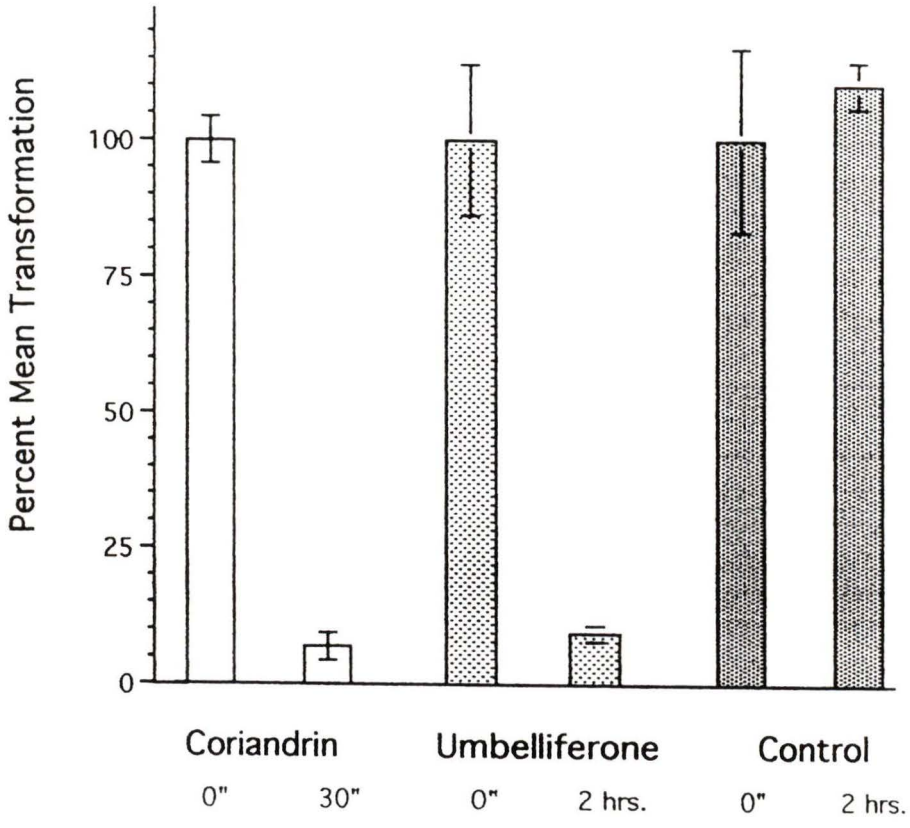
One final transformation experiment for umbelliferone activity was done with both positive and negative controls present. DNA was treated with coriandrin at 5 μg/ml, umbelliferone at 15 μg/ml or 0.1% DMSO control with and without NUV and 4.0 ng then used to transform Epicurian Coli SURE Cells™. Results are depicted in fig. 35. Numbers of transformant colonies were not significantly different for coriandrin/without NUV, umbelliferone/without NUV, and control/without NUV when compared to each other. ($p = 0.145$, $p = 0.411$, $p = 0.663$) Numbers obtained for each dark control was used as 100%. Mean percentage of transformants dropped to 6.9% for coriandrin/30 seconds NUV (402 Joules/m^2) and 9.4% for umbelliferone/2 hrs. NUV (96.48 kJ/m^2). Both sets of data were significant at the $p < 0.0005$ level. Numbers of transformants did not change significantly between control DNA with and without 2 hrs. NUV going from a mean of 278.3 transformants to a mean of 308. Photographs of representative plates are given in fig. 36.

Figure 34. Transformation of Epicurian Coli SURE ™ cells by pTZ18R treated with or without NUV light in the presence of either 5 or 15 µg/ml of umbelliferone or 5 µg/ml coriandrin. 4.0 ng of plasmid DNA was used for each transformation procedure. NUV irradiation was performed on plasmid for 0 seconds, 30 seconds, 1 hr. or 2 hrs. as indicated (0, 0.40, 48.24, or 96.48 kJ/ m^2) and then appropriate aliquots added to cells in described transformation procedure. Transformations were done with Lot #2 cells.



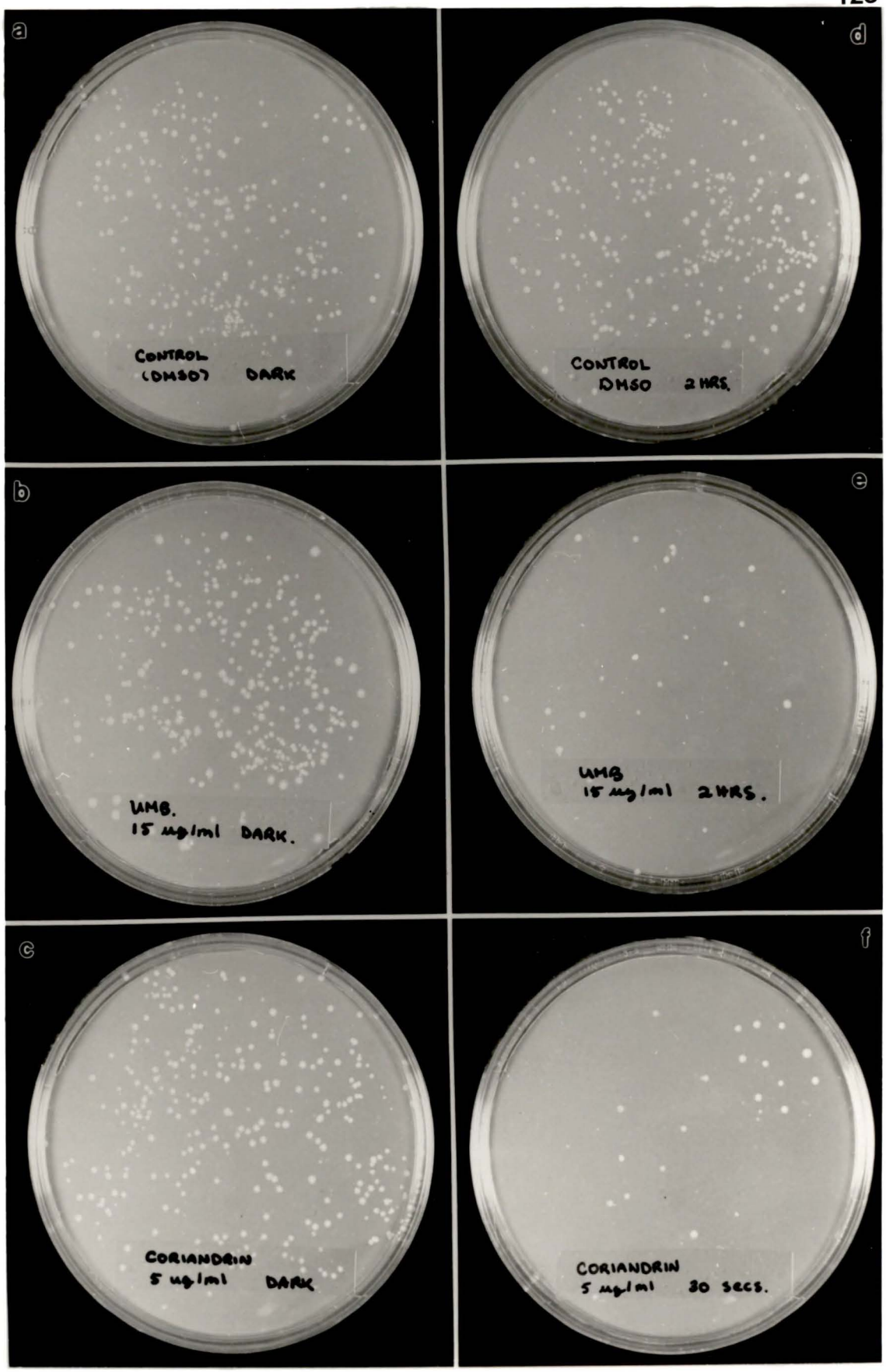
Standard Deviation Bars shown

Figure 35. Transformation of Epicurian Coli SURE ™ cells by pTZ18R treated with or without NUV light in the presence of either 0.1% DMSO (Control), 15 μg/ml of umbelliferone or 5 μg/ml coriandrin. 4.0 ng of plasmid DNA was used for each transformation procedure. NUV irradiation was performed on plasmid for 0, 30 seconds, or 2 hrs. as indicated in the presence of compound and millipore water only (0, 0.40, or 96.48 kJ/ m^2) and then appropriate aliquots added to cells in described transformation procedure. Lot #2 cells were used.



Standard deviation bars shown

Figure 36. Photographs of transformation of Epicurian Coli SURE ™ cells by pTZ18R treated with either 0.1% DMSO (a and d), 15 µg/ml umbelliferone (b and f), or 5 µg/ml coriandrin (c and f) described in fig. 35. a, b, and c plates were from transformation with plasmid treated with compound only while d, e, and f were plasmid treated with both compound and NUV light. d and e were exposed to 96.48 kJ/ m^2. f was exposed to 402 Joules/ m^2 of NUV.


a
CONTROL
(DMSO) DARK
d
CONTROL
DMSO 2 HRS.
b
UMB.
15 µg/ml DARK.
e
UMB
15 µg/ml 2 HRS.
c
CORIANDRIN
5 µg/ml DARK
f
CORIANDRIN
5 µg/ml 30 secs.

Irradiation of umbelliferone

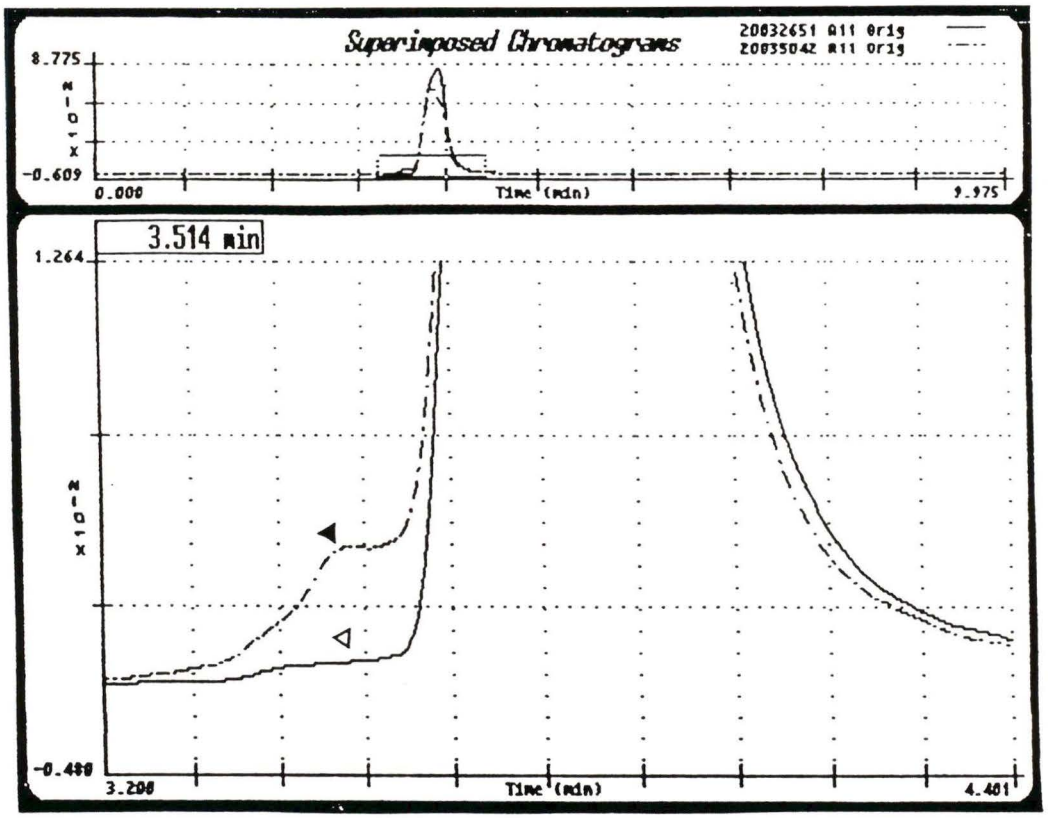
As stated in the characterization results section, umbelliferone would convert to 2-5 different products in the presence of light depending on the particular conditions. Impurities/photoproducts which were present without light irradiation of the compound showed a yellow fluorescence under UV at Rf value 0.00 and 0.30 would increase in quantity if umbelliferone was irradiated with NUV as shown by figs. 9, 10b and c. Irradiation with NUV on TLC would also produce five new photoproducts at Rf values of 0.55-0.58 (yellow), 0.61-0.63 (blue), 0.65-0.67 (blue), 0.72-0.733 (blue) and 0.77-0.79 (yellow/blue) as shown by figs. 10b and c, and 15. (Colors in brackets were fluorescence as seen under UV). All photoproducts would not form all the time and appeared to be dependent on amount of umbelliferone spotted and area of irradiation. This is clearly demonstrated in fig. 10c when three photoproducts formed from initial irradiation and then 5 from re-irradiation after 1D TLC.

Crystals irradiated on glass produced similar photoproducts with Rf values of 0.61, 0.72, and 0.76 but comparatively less than silica TLC irradiation as shown by fig. 17. The photoproduct from irradiation on glass with Rf value 0.60, although with same Rf value as on silica, fluoresced yellow/blue rather than yellow but the quantity was very faint so it was difficult to discern color (see figs. 10b and c, 15 and 17). Irradiated umbelliferone in phosphate buffer and agar used in described biological assays also changed to a light yellow color with NUV or sunlight. Crystals irradiated in glass were also analyzed by HPLC as shown by fig. 37. Standard umbelliferone had a retention time of 3.91 mins. while the photoproducts had a retention time of 3.58 mins. and were 2.97% of the sample area.

As stated earlier and as shown by fig. 10c, these products also appeared to be able to convert from one to another when re-irradiated. After spotting umbelliferone and irradiation under NUV for 96.48 kJ/m^2, products were separated in the first direction and

Figure 37. Reverse phase high performance liquid chromatogram of umbelliferone before and after irradiation. Sample concentration analyzed at 40 µg/ml in mobile phase and using a Beckman 126 pump, 166 detector and System Gold integration software. Samples run on 10 mm x 25 cm ultrasphere ODS semi-preparative column with 5 µm pore size and 50%/50% CH_3CN/ water and detection wavelength set at 254 nm. Flow rate= 3 ml/min.

a) Standard umbelliferone Rt = 3.91 mins., 100% area
b) Irradiated umbelliferone Rt = 3.84 mins., 97.04% area
photoproduct Rt = 3.58 mins., 2.97% area



Irrad. Umb ▼
Non-irrad. Umb ▽

visualized with UV. These spots were re-irradiated for an equal amount of time and then run in the 2nd direction in the same solvent. As can be seen by fig. 10c, the compound at the origin partially reconverted back to umbelliferone while the yellow and blue spots of Rf value 0.61 and 0.67 also partially reconverted back to umbelliferone. Standard umbelliferone, as shown by fig. 10a and b, which had bound products at the origin also reconverted back to umbelliferone.

An attempt was made to characterize these photoproducts. After TLC separation, scraping, and extraction, these compounds appeared to revert back to umbelliferone in a similar fashion as the yellow impurity isolation depicted in fig.9. A mixture was also sent for mass spectrometry but the products were of insufficient yield for analysis. Absorbance measurements of the mixture showed a decrease in absorbance of umbelliferone at 325 nm but because of the broad 325 nm peak of umbelliferone and insufficient production of compound, characteristic absorbance of the photoproducts was not detected.

Absorption spectra of umbelliferone in phosphate buffer were taken at pH 6.8 and 7.8 before and after 25 and 85 mins. of NUV irradiation (20.1 and 68.24 kJ/m^2) (see fig. 38 and 39). Fig. 39 shows formation of photoproducts which absorb in the 495 nm range in pH 7.8. These products were also noted at pH 6.8 but at lesser quantities. No other new peaks were noted. A decrease in absorbance at 325 nm (maxima for protonated form) and 367 (maxima for anion) was also noted with increasing radiation dose that was more marked for pH 7.8 than 6.8 as shown by fig. 40.

Fluorescence spectrophotometry also showed a decrease at the characteristic fluorescence of umbelliferone at 460 nm with increasing irradiation dose as shown by fig. 41 and 42. Irradiation procedures and dosages of 0, 25, and 85 mins. of NUV were performed identical to those done for absorption study. This decrease was again greater at pH of 7.8 than 6.8 (330 nm and 367 nm

Figure 38. Absorption spectra of umbelliferone at 40 µg/ml in Sorrensen's phosphate buffer (0.07 M) at pH 6.8 (a) and pH 7.8 (b) (Beckman DU-64 spectrophotometer, scan speed 500 nm/min.).

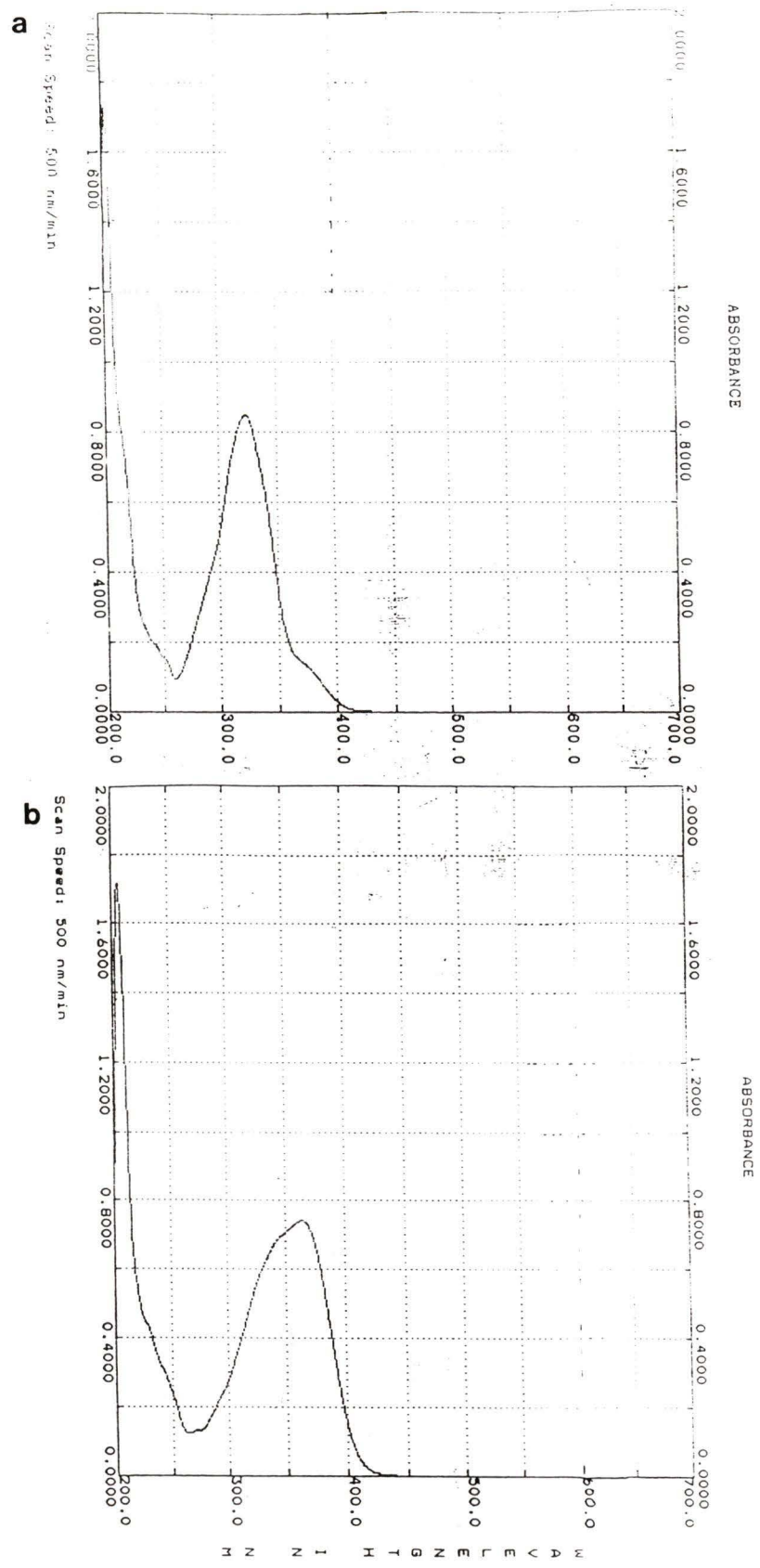

a
b
Scan Speed: 500 nm/min
ABSORBANCE
WAVELENGTH nm
200.0
300.0
400.0
500.0
600.0
700.0
0.0000
0.4000
0.8000
1.2000
1.6000
2.0000

Figure 39. Absorption spectra of umbelliferone between 375 and 550 nm at 40 µg/ml in Sorrensen's phosphate buffer (0.07 M) at pH 7.8 before and after NUV irradiation. a) non-irradiated control b) irradiated x 68.34 kJ/ m^2 (Beckman DU-64 spectrophotometer, scan speed 500 nm/min.).

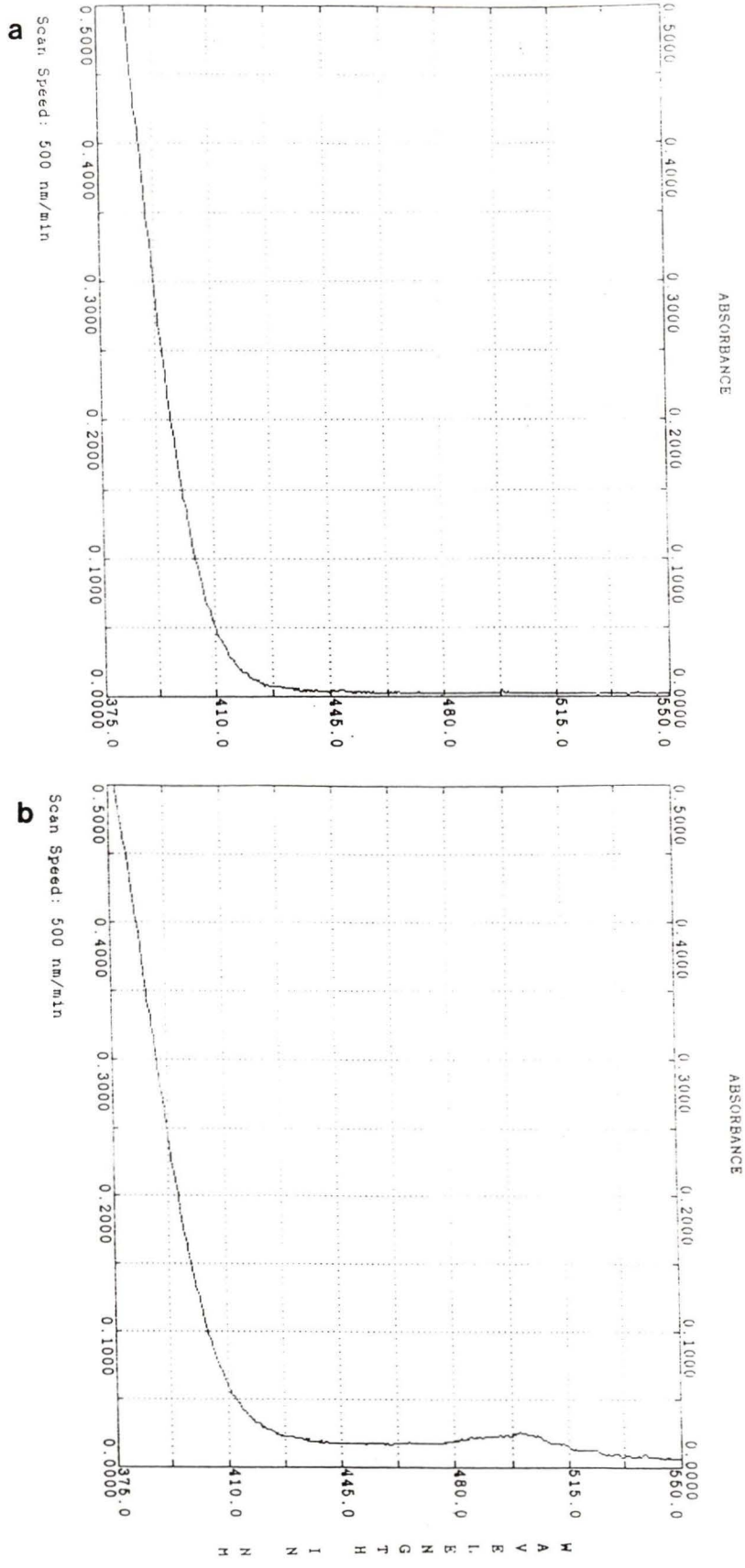

a
Scan Speed: 500 nm/min
ABSORBANCE
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
375.0
410.0
445.0
480.0
515.0
550.0
b
Scan Speed: 500 nm/min
ABSORBANCE
WAVELENGTH IN NM

Figure 40. Absorbance at 325 and 367 nm of umbelliferone irradiated in 0.07 M phosphate buffer at pH 6.8 and 7.8. NUV irradiation was performed on 5 mls of solution in a 70 ml tissue culture flask with liquid depth < 2 mm for 0, 20.1, and 68.34 kJ/ m^2. Conditions of irradiations was identical to those performed for fluorescence spectra in fig.41. Absorbance decrease was greater for pH 7.8 (84.7%) than for pH 6.8. (90.0%). No statistical significance is given as insufficient sampling was done. Absorbance maxima for protonated form of umbelliferone is 325 nm while the maxima for the anion is 367 nm.

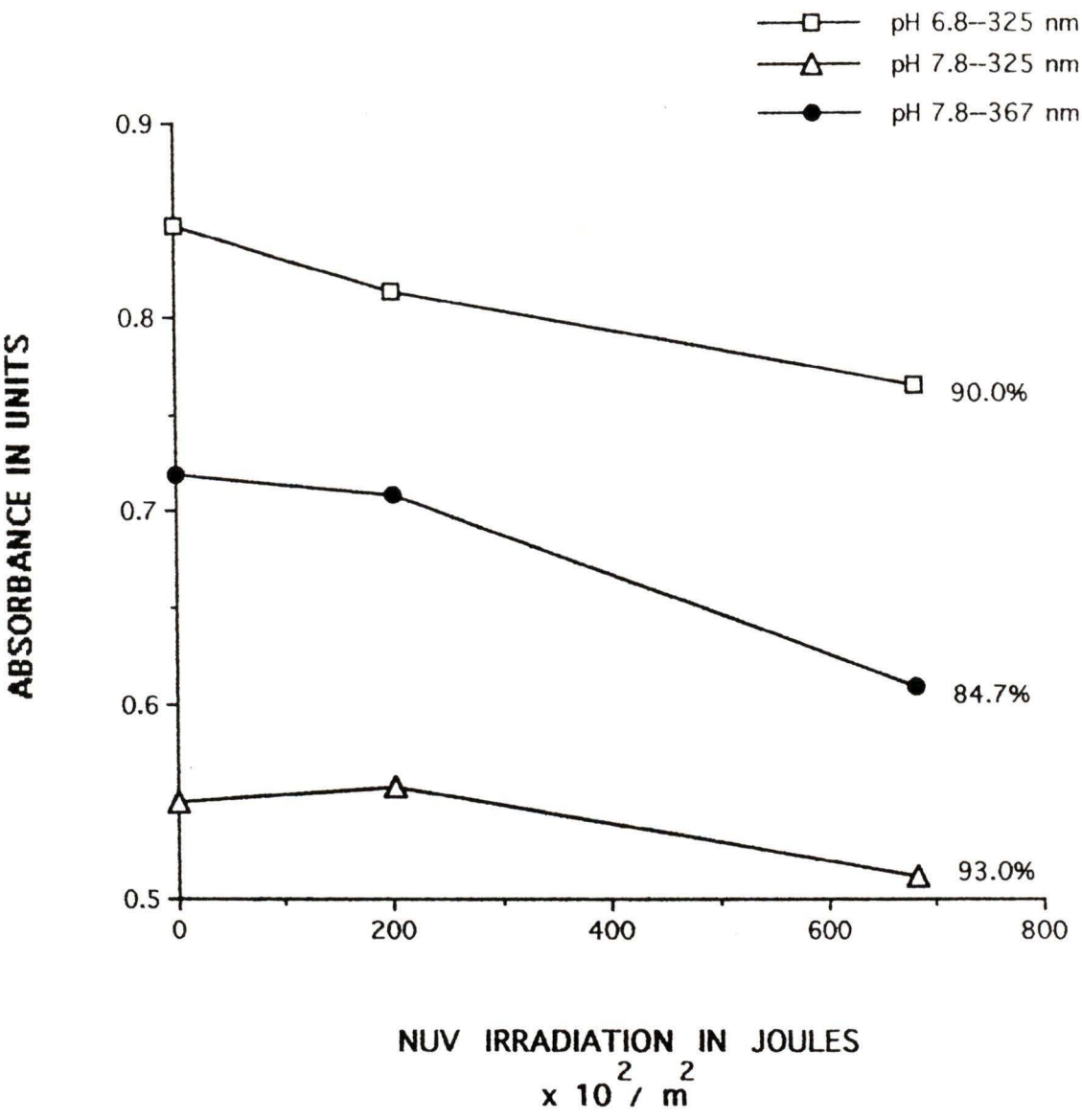

pH 6.8--325 nm
pH 7.8--325 nm
pH 7.8--367 nm
0.9
0.8
0.7
0.6
0.5
0
200
400
600
800
90.0%
84.7%
93.0%
ABSORBANCE IN UNITS
NUV IRRADIATION IN JOULES
x 10 2 / m 2

Figure 41. Fluorescence spectra of umbelliferone (approximately 0.04 µg/ml: serial dilution of a 40 µg/ml stock) in 0.07 M Sorrensen's phosphate buffer on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at two different pH's: 6.8 and 7.8 using an excitation wavelength of 330 nm and slit widths of 2/2 nm after 0, 25 and 85 mins. of NUV irradiation (0, 20.1, 68.34 kJ/ m^2).

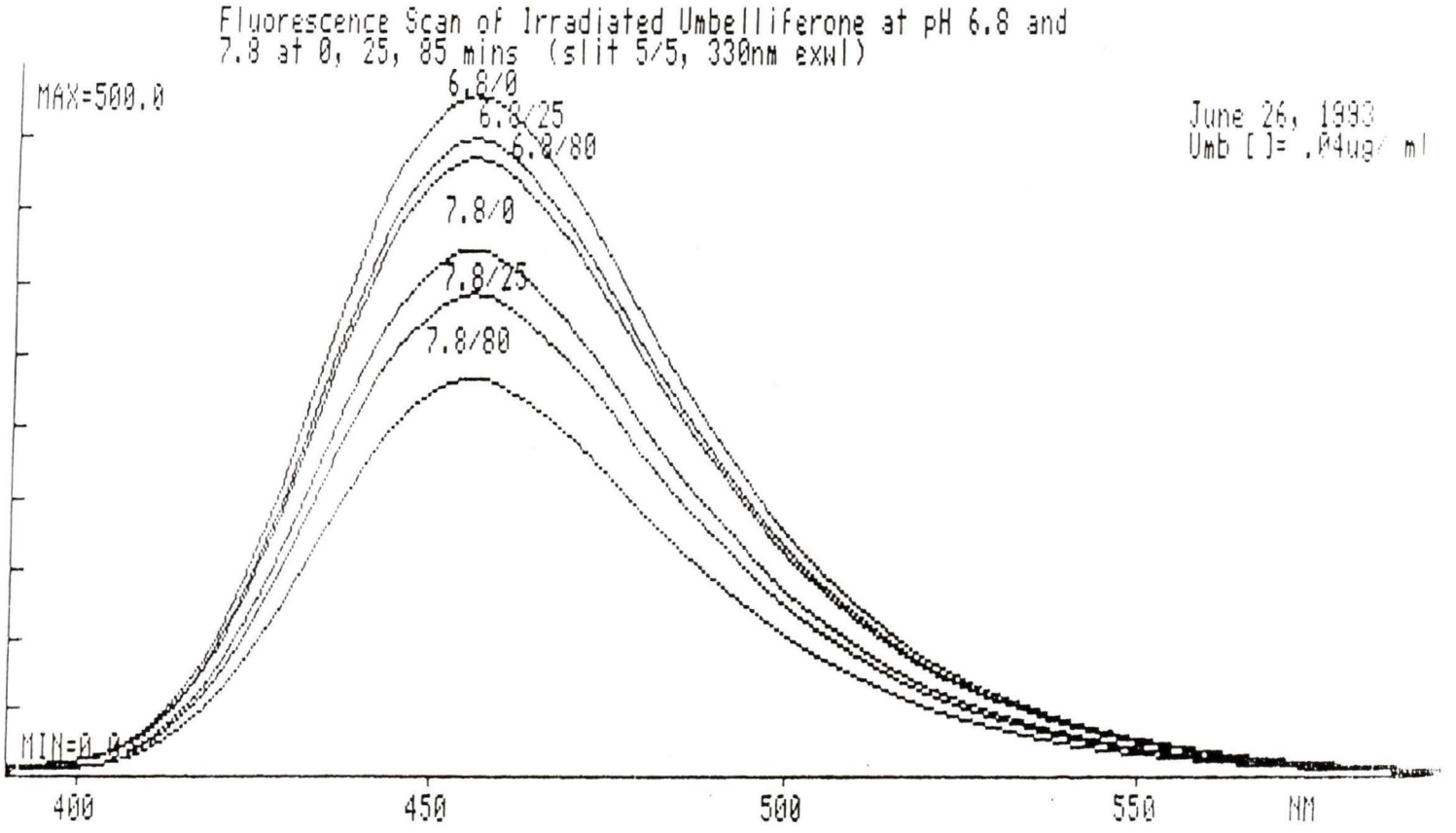
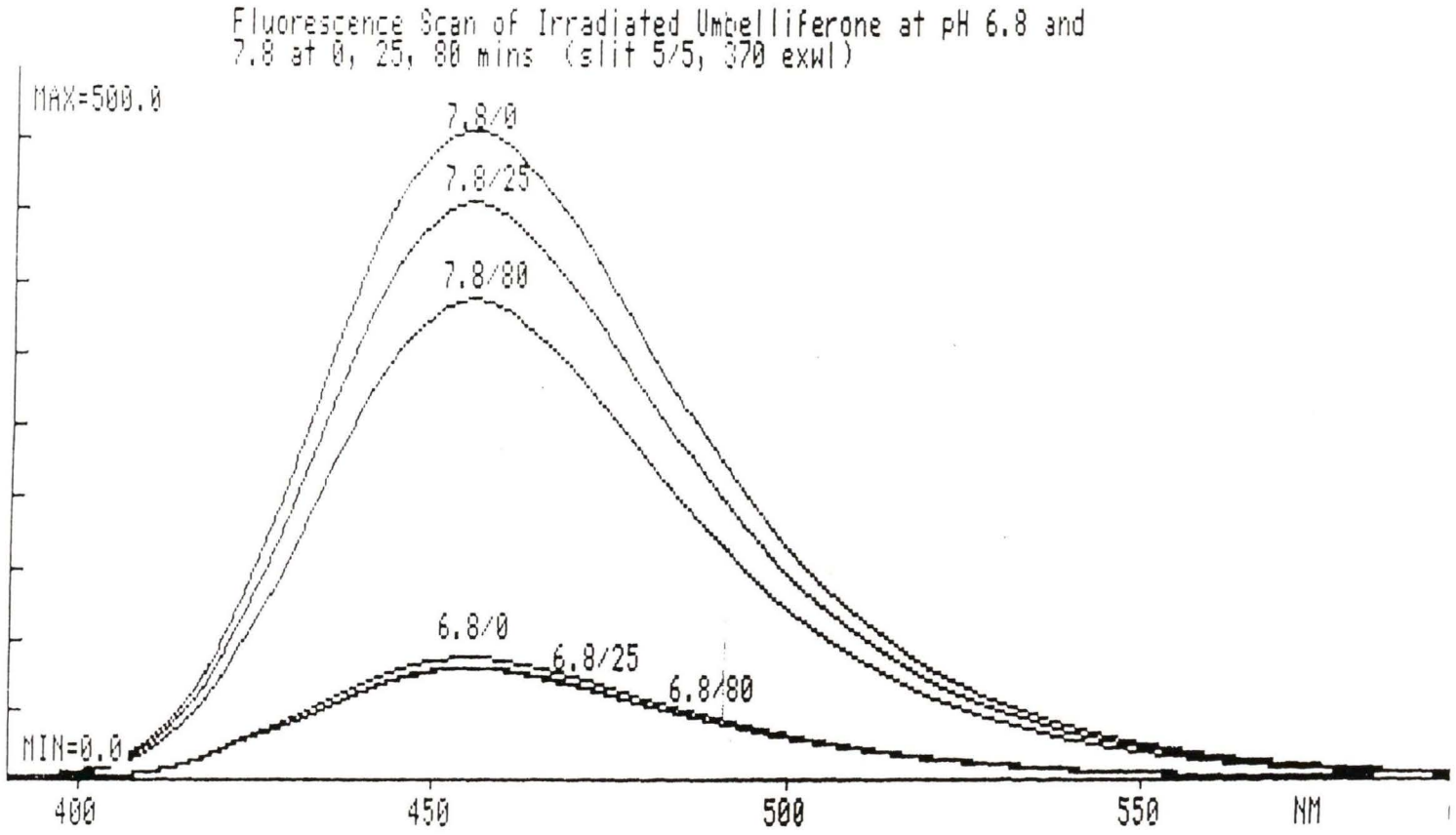

Fluorescence Scan of Irradiated Umbelliferone at pH 6.8 and 7.8 at 0, 25, 85 mins (slit 5/5, 330nm exwl)
MAX=500.0
MIN=0.0
6.8/0
6.8/25
6.8/80
7.8/0
7.8/25
7.8/80
June 26, 1983
Umb [ ]= .04ug/ ml
400
450
500
550
NM

Figure 42. Fluorescence spectra of umbelliferone (approximately 0.04 µg/ml: serial dilution of a 40 µg/ml stock) in 0.07 M Sorrensen's phosphate buffer on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at two different pH's: 6.8 and 7.8 using an excitation wavelength of 367 nm and slit widths of 2/2 nm after 0, 25 and 85 mins. of NUV irradiation (0, 20.1, 68.34 kJ/ m^2).


Fluorescence Scan of Irradiated Umbelliferone at pH 6.8 and 7.8 at 0, 25, 80 mins (slit 5/5, 370 exwl)
MAX=500.0
MIN=0.0
7.8/0
7.8/25
7.8/80
6.8/0
6.8/25
6.8/80
400
450
500
550
NM

excitation wavelengths were used for the neutral and anion forms respectively). Again, because of the broad fluorescence spectrum of umbelliferone, it was difficult to pick out any characteristic fluorescence of photoproducts without isolation. No changes could be seen in 0.8% ethanol control after irradiation in absorption or fluorescence spectra.

Absorption spectra of umbelliferone irradiated in different solvents and on glass also showed a decrease in its characteristic 325 nm absorption. 200 µg of umbelliferone was placed on a watch glass or dissolved in water, 95% ethanol or methanol and irradiated for NUV for 96.48 kJ/ m^2. The greatest decrease occurred in 95% ethanol with the absorbance 27.6% less than the average absorbance of all others as shown by fig. 43. 1D TLC analysis with hexane/acetone 1:1 showed formation of varied products depending on the solvent used (see fig. 44). Concurrent development of 7-ethoxy and 7-methoxycoumarin showed that newly formed products had different Rf values. An attempt was made again to identify these products by sending a mixture to mass spectrometry. However, either through low product yield or reconversion back to umbelliferone, identification of compounds was not possible. An attempt was also made to characterize products formed when irradiating umbelliferone in methanol by NMR by Dr. Peter Wan at the University of Victoria Chemistry Department but this was also unsuccessful. 2D TLC analysis showed the instability of photoproducts as they would convert back and forth during development as shown by fig. 45.

Irradiation of umbelliferone in sunlight and fluorescent lights was also investigated. After irradiation in phosphate buffer, the umbelliferone solution fluoresced a bright yellow orange in visible light and under UV. This also occurred when umbelliferone was irradiated by the sun on TLC. This yellow product would not move on TLC from area of irradiation until very polar solvents such as 80:1 methanol/water were used (see fig. 46). Standard umbelliferone, as

Figure 43. Absorbance at 325 nm for equal amounts of umbelliferone irradiated in different conditions. Absorbance decreased the most for irradiation in ethanol having a value of 27.6% less than the average of the absorbance of control umbelliferone (Std. Umb.) and that irradiated in different conditions. All samples, including control, were evaporated after irradiation and then redissolved in methanol to a concentration of 20 μg/ml. Absorbance was taken on a Beckman DU-64 spectrophotometer with a 1 cm pathlength. Samples were as follows: irradiated in millipore water, on glass, (Irrad. alone), irradiated in 95% ethanol (Irrad. EtOH), and irradiated in methanol (Irrad. MeOH).

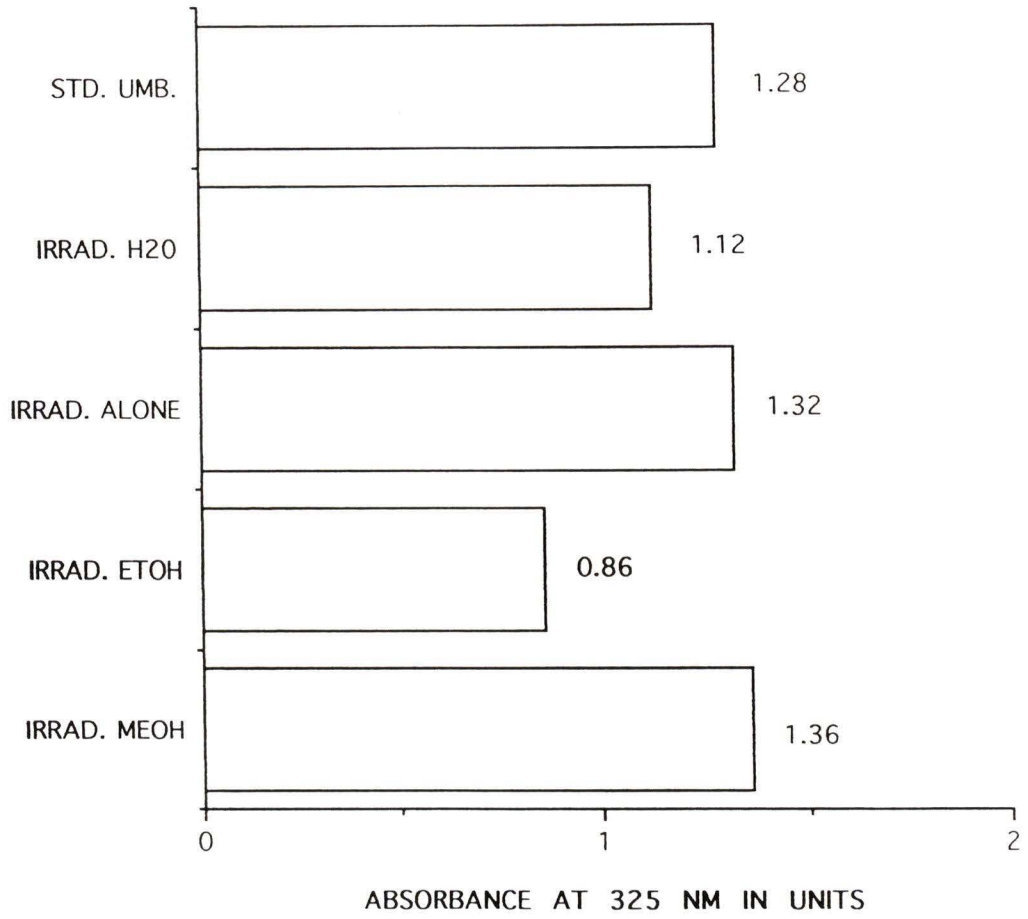

STD. UMB.
1.28
IRRAD. H20
1.12
IRRAD. ALONE
1.32
IRRAD. ETOH
0.86
IRRAD. MEOH
1.36
0
1
2
ABSORBANCE AT 325 NM IN UNITS

Figure 44. 1D silica TLC of umbelliferone irradiated in methanol, and irradiated in 95% ethanol as compared to 7-methoxycoumarin, 7-ethoxycoumarin and a 95% ethanol and a methanol standard of umbelliferone (20 μg of each spotted) (hexane/acetone 1:1 development). Solvent front is indicated by line marked with an s.
Symbols: IM= irradiated in methanol, IE= irradiated in 95% ethanol, 7M= 7-methoxycoumarin, 7E= 7-ethoxycoumarin, Std. E= umbelliferone standard in 95% ethanol, Std. M= standard in methanol. Color symbols: y= yellow, b= blue, v=violet as seen under UV.

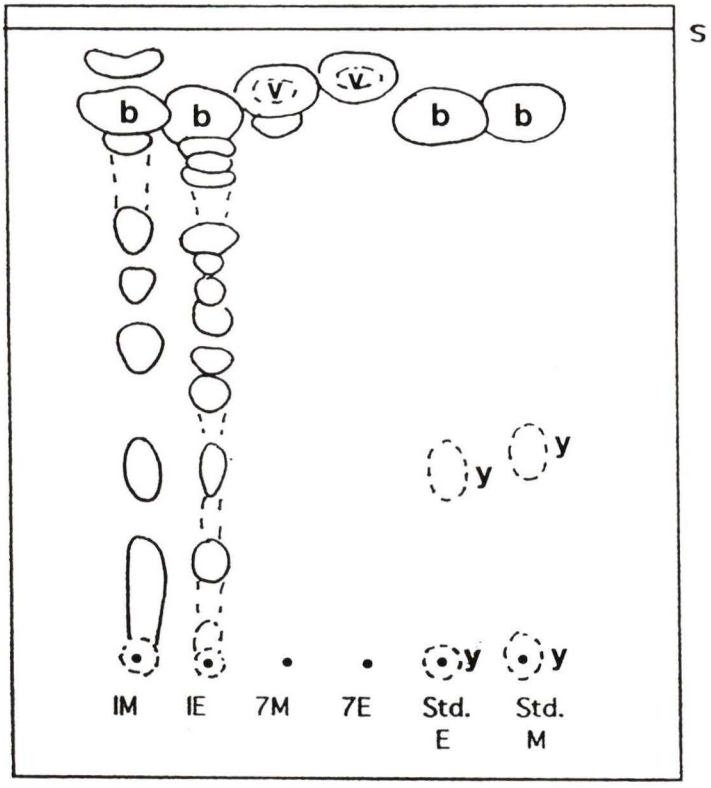

S
b
b
V
V
b
b
y
y
y
y
IM
IE
7M
7E
Std. E
Std. M

Figure 45. 2D silica TLC of irradiated umbelliferone in solutions of a) 95% ethanol and b) methanol. Development of compounds in hexane/acetone 1:1 in both directions. Compound conversions during TLC process are highlighted by shading (30 µg spotted). Solvent fronts are indicated by lines marked with s. The symbol b= blue as seen under UV and indicates position of umbelliferone. Fluorescence of umbelliferone was stronger than all other compounds.

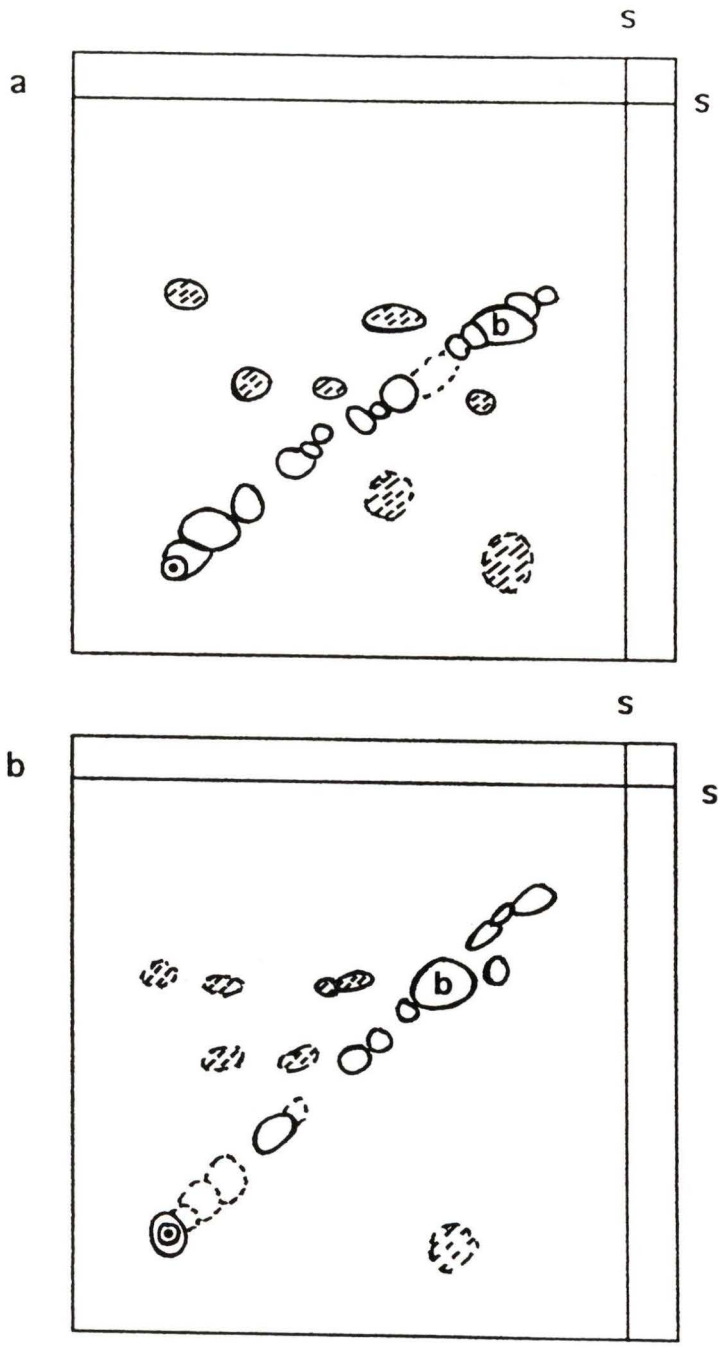

a
s
s
b
b
s
s

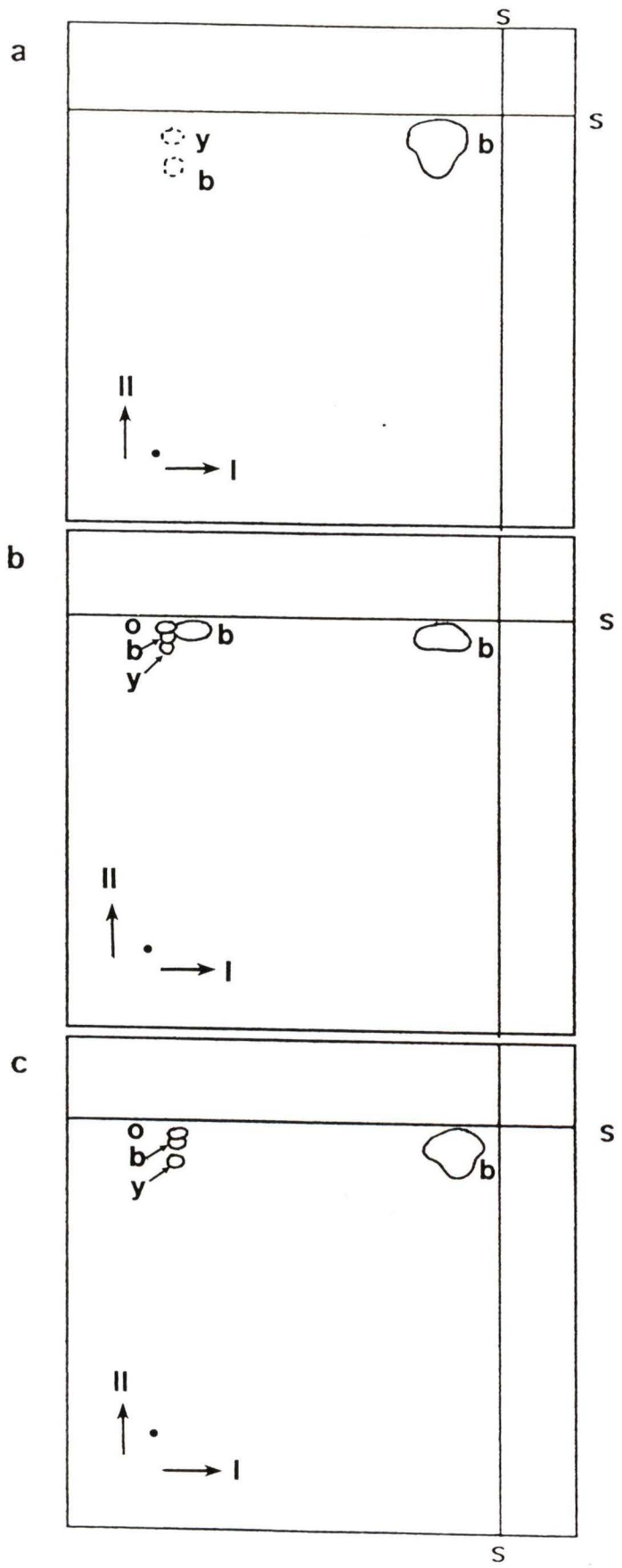
Figure 46. 2D silica TLC of irradiated umbelliferone in sunlight for 10 hours and fluorescent lab lights for 24 hrs. (in July, 1993 in Victoria, BC). TLC's were developed in first direction with solvent I) hexane/acetone 1:1 and in second direction with solvent II) methanol/water 80:1 (30 μg spotted). Solvent fronts indicated by lines marked with an s.

a) umbelliferone Rf in I = 0.88, Rf in II= 1.00
yellow impurity Rf in I = 0.00, Rf in II= 0.92
blue impurity Rf in I = 0.00, Rf in II= 0.84

b) sun irradiated umbelliferone
blue photoproduct Rf in I = 0.14, Rf in II= 0.96
dark yellow photoproduct Rf in I = 0.00, Rf in II = 0.96
blue photoproduct II Rf in I = 0.00, Rf in II = 0.93
yellow photoproduct Rf in I = 0.00, Rf in II = 0.90

c) umbelliferone irradiated with fluorescent lights.
photoproducts have all Rf value = 0.00 in solvent I
dark yellow photoproduct Rf in II = 0.95
blue photoproduct Rf in II = 0.92
yellow photoproduct Rf in II = 0.87

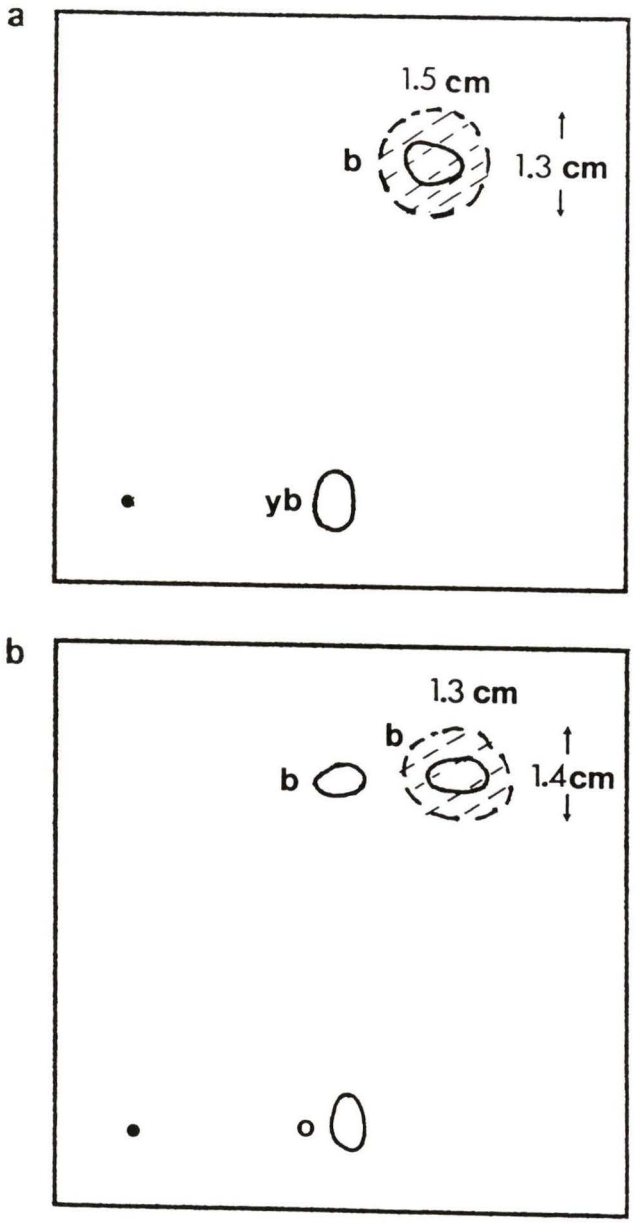
Colours are fluorescence as seen under UV: b= blue, o=dark yellow, y= yellow


a
S
S
y
b
b
II
I
b
S
o
b
y
b
b
II
I
c
S
o
b
y
b
II
I
S

depicted by fig.46a, had impurities at the origin left behind by hexane/acetone 1:1 (solvent I) which had an Rf value of 0.92 (yellow) and 0.84 (blue) in methanol/water 80:1 (solvent II). Umbelliferone exposed to sunlight on TLC for one full day (July '93 in Victoria, BC) had a new blue product at 0.14 in solvent I and 0.96 in II. Umbelliferone on TLC exposed to sunlight and fluorescent lights also had a new dark yellow product at Rf 0 in I and 0.95-6 in II. Impurities which were present in the standard were present in greater quantities in both irradiated samples. These very polar photoproducts formed by the sun were tested for photosensitizing ability against *E. coli B/s* $_{-1}$, as shown in fig. 47, but were negative.

Thus, characterization of umbelliferone's photoproducts was not possible via NMR or mass spectrometry but it was shown conclusively that umbelliferone was converting into 2-5 products under NUV and sunlight. All showed a yellow or blue fluorescence under UV and some had a 495 nm absorbance. The yield and type of products seemed to vary with irradiation dose, at different pH, on TLC versus glass, and when irradiated in different solvents.

Figure 47. 5 µg of umbelliferone was spotted and developed half way in one direction with hexane/acetone 1:1. TLC plates were left in a) dark or b) sun for 10 hrs. in July, 1993 in Victoria, BC and then redeveloped in 2 directions with above solvent. A significant amount of highly fluorescent dark yellow compound was left at the point of compound irradiation but was not photoactive. Inhibition zones on photobioassay with *E. coli B/s* $_{-1}$ are shown by shading. Some of the dark yellow compound showed reconversion into a blue product with an Rf value equal to umbelliferone. Colours are fluorescence as seen under UV: b= blue, or yb= yellow and blue, o= dark yellow. Measurements given are the width of the inhibition zones at the widest points in both vertical and horizontal directions.


a
1.5 cm
b
1.3 cm
yb
b
1.3 cm
b
b
1.4cm
o

Discussion

Purity of umbelliferone

Establishing the purity of umbelliferone was extremely important as isopimpinellin (5,8-dimethoxypsoralen), thought previously to be photobiologically active, was later found to be inactive with positive results attributed to an impurity (Ashwood-Smith et al., 1992). Isopimpinellin obtained from plant extracts often has small amounts of the very active furanocoumarin, 5-methoxypsoralen as an impurity. 5-methoxypsoralen is difficult to separate from isopimpinellin as it runs very close to isopimpinellin on both TLC and HPLC in many resolutions (Ashwood-Smith et al., 1992). Isopimpinellin, thus, had conflicting reports of photoactivity; but using synthesized pure isopimpinellin in our laboratory, it was later found to be photobiologically inactive. As shown by Table 1, umbelliferone has also had conflicting reports of photoactivity and thus, extensive analysis on TLC and HPLC was done to ensure that no contaminant was present. It was also important to confirm that our compound was umbelliferone and therefore, umbelliferone's characteristics were confirmed by NMR, mass spectrometry and melting point.

Umbelliferone first appeared to have minor impurities on TLC and occasionally on HPLC as shown by figs. 5 and 6 that did not alter after repeated crystallizations in water or toluene. Attempts to purify the compound on TLC through development and extraction of umbelliferone were also unsuccessful. Other tests such as melting point, HPLC resolutions at other various detector wavelengths and mobile phases, and gas chromatography showed that umbelliferone was essentially quite pure as shown by figs. 7 and 8. The melting point of umbelliferone was also not depressed; however, the impurity may have sublimed during testing.

It was later found that umbelliferone converted to other products in the presence of light and that these "impurities" on TLC and HPLC

were most likely photoproducts of umbelliferone. As detailed following, umbelliferone also appeared to convert to different types and amounts of photoproducts depending upon the wavelength of irradiation and the conditions of irradiation.

Irradiation of umbelliferone increases the amount of impurity shown on TLC which converts back to umbelliferone upon isolation attempts

As shown by fig. 5, recrystallizations of umbelliferone had no effect on amount of impurities present at Rf 0.00 and 0.30 on TLC. Fluorescent laboratory lights, sunlight, and NUV irradiation of umbelliferone spotted on TLC increased the amount of impurities detected originally on TLC as shown by figs. 10c, 46 and 47. Fig. 10c shows that irradiation of umbelliferone after 1D development increased the amount of product left at the origin. Figs. 46 and 47 also show an increase of product left at the origin after irradiation with either fluorescent laboratory lights or sunlight. The impurity at Rf 0.30 also occasionally increased slightly with irradiation but data is not shown.

The increased "impurities" at Rf value of 0.00 and occasionally at 0.30 could not be isolated through TLC development, extraction, and redevelopment. During the isolation procedure, as shown by fig. 9, the impurity at Rf 0.30 converted to a compound with an Rf value equal to that of umbelliferone. Isolation of impurities on TLC by scraping of TLC and extraction with methanol followed by injection into HPLC also showed a peak with a retention time equal to that of umbelliferone (data not shown). The impurity left at the origin also behaved in a similar fashion in both TLC and HPLC methods. This behaviour is again demonstrated during a 2D TLC development in figs. 10 a, b and c. In each figure, compound left at the origin after development in one direction partially converts to a compound with an Rf value equal to that of umbelliferone during development in the second direction. Overloading was not a factor as no tailing of compounds were noted. The amount of impurity left at the origin

appeared dependent on the surface area of the umbelliferone spot and influence of irradiation. For example, fig. 10c shows the highest amount of impurity at Rf 0.00 after irradiation of a spot that has been spread by 1D development. In comparison, the spot left at the origin in fig. 10b is smaller than in 10c but greater than what is left in the standard in fig. 10a. Another example is demonstrated by the extent of the inhibition zone by the compound at the origin in fig. 10d compared to 10e.

These factors of an increase in "impurities" with irradiation and conversions of impurities back to umbelliferone during isolation procedures strongly suggest that these impurities are not impurities at all but photoproducts or conversion products of umbelliferone. These conversions may be catalyzed by both light and binding to silica as their production increased with an increase in the surface area of umbelliferone bound to TLC.

Irradiation of umbelliferone increases the amount of impurity shown on HPLC

In further support of the idea that impurities found are actually photoproducts of umbelliferone, irradiation of umbelliferone crystals on glass increased the amount of impurity detected originally on HPLC. As shown by fig. 6, the impurity detected on HPLC was not affected by repeated recrystallizations in either toluene or water. As shown by fig. 37, the impurity originally detected on HPLC increased in concentration after umbelliferone crystals were irradiated with NUV on a watchglass.

Thus, as impurities detected on TLC and HPLC appeared to be photoproducts of umbelliferone and not true contaminants, any photoactivity found on biological assays could be attributed to umbelliferone or its photoproducts and not to extraneous impurities.

Irradiation of umbelliferone in different conditions produces different amounts and types of photoproducts

Extensive further testing of umbelliferone irradiated in different conditions were done to support the above hypothesis that umbelliferone could produce photoproducts. Umbelliferone was irradiated after absorption onto TLC, in different solutions, on glass and using different wavelengths of light for different times to investigate the types and quantities of photoproducts produced.

i) Irradiation of umbelliferone on silica TLC versus glass

TLC plates appeared to catalyze greater formation of photoproducts than irradiation on glass as shown by the amount and number of products depicted in fig. 10b and c compared to fig. 17. Numerous new photoproducts on TLC appeared which had Rf values of 0.00 (yellow), 0.58 (yellow), 0.67 (blue), 0.73 (yellow/blue) and 0.79 (blue) shown in fig.10c. Only 3 photoproducts were obtained from glass irradiation but had similar Rf values to those obtained from irradiation on TLC: 0.61, 0.72, and 0.76. Two photoproducts, at 0.58 (yellow) and 0.67 (blue) from irradiation on TLC also appeared to convert back to umbelliferone with an Rf of 0.88 after re-irradiation as shown by fig. 10c. Thus, umbelliferone absorbed onto TLC appeared to catalyze its conversion into other products better than crystals alone on glass and also showed interconversions of photoproducts back into umbelliferone. Also, umbelliferone photoconversion appeared to not be an artifact from a combination of irradiation and binding to TLC only as photoproducts could be detected from irradiation on glass as well.

ii) Irradiation of umbelliferone in solution

Irradiation of umbelliferone in solution also showed photoconversion into other products. Irradiation with NUV of umbelliferone in phosphate buffer showed a yellow coloration of the solution in visible light compared to a clear control. Umbelliferone

was shown to degrade in phosphate buffer via a decrease in absorbance at 325/367 nm and fluorescence at 460 nm as shown by figs. 40, 41 and 42. This decrease behaved in a dose dependent manner and resultant new photoproducts absorbed at 495 nm as shown in fig. 39. Umbelliferone breakdown appeared to be greater in pH 7.8 than 6.8 as shown by greater decreases in absorbance and fluorescence.

Irradiation of umbelliferone in different solvents such as ethanol and methanol also showed photodegradation of umbelliferone and production of different photoproducts as shown by fig. 44. These new products were not due to production of 7-ethoxy or 7-methoxycoumarin as exhibited by the different Rf values. These new compounds, like those produced in the 2D analysis of repeatedly NUV irradiated umbelliferone in fig. 10c, showed instability and interconversions during a 2D TLC (see fig. 45) as shown by the different Rf values in the same solvent. As shown by fig. 45, instead of all compounds falling on a diagonal line from a 2D TLC with the same solvent, compounds indicated with shading have changed during the TLC development. Product conversion due to interaction with the silica on the TLC itself was not ruled out. Umbelliferone breakdown seemed to be greatest in 95% ethanol as shown by the greatest decrease in 325 nm absorbance as shown by fig. 43. This difference, however, was not statistically significant. Attempts were made to characterize these products but were unsuccessful due to either low yield of products or conversion of photoproducts back to umbelliferone during analysis.

iii) Irradiation of umbelliferone with different wavelengths of light

Umbelliferone also appeared to convert to different photoproducts depending on the type of irradiation source. Fluorescent laboratory lights and sunlight greatly increased the formation of impurity/photoproducts bound to the origin on TLC (see fig. 46). Both sunlight and fluorescent laboratory lights formed a new dark yellow product with an Rf of 0.00 in hexane/acetone 1:1

(solvent I) and an Rf of 0.95-0.96 in methanol/water 80:1 (solvent II). Sunlight also formed a new blue photoproduct with an Rf value of 0.14 in solvent I and 0.96 in solvent II. None of these photoproducts were formed by irradiation by NUV on TLC as shown by fig. 10b. Sunlight and fluorescent laboratory lights also turned solutions of umbelliferone at 40 μg/ml in phosphate buffer dark yellow and light yellow respectively as seen under visible light when compared to a control. This coloration was similar to that produced by 2 hours of NUV but was darker for sunlight and lighter for fluorescent lights.

Our NUV lights have a large broad peak at 310-380 nm with minor peaks at 400 and 440 nm (Ashwood-Smith et al., 1992). Fluorescent lights in our laboratory (380-680 nm) were shown to have identical peaks at 400 and 440 nm as our NUV lights (Ashwood-Smith et al., 1992). These fluorescent lights were shown in our laboratory to photodegrade 5-geranoxypsoralen and it was postulated that this overlap of emission spectra may help to explain why both NUV and fluorescent room lights cause degradation of 5-geranoxypsoralen (Ashwood-Smith et al., 1992). This may also be the case for umbelliferone photoconversion. Activating wavelengths for photoconversion of umbelliferone were not determined although the most likely ranges are around 325 and 367 nm which are the wavelengths at which umbelliferone most strongly absorb. Our fluorescent laboratory lights tail off in the 380 nm range and as shown by the absorption spectrum in fig. 48 and Fink & Koehler (1970), umbelliferone does absorb in this range. Photoproducts of umbelliferone have been shown in this study to absorb in the visible portion of the light spectrum (495 nm) (see fig. 39) and may undergo further conversions using these wavelengths found in our fluorescent lights and sunlight.

As shown by the above data, umbelliferone without question converted to other products in the presence of light when absorbed onto TLC, on glass and when dissolved in solution. Depending on the conditions of irradiation and the wavelengths of light used,

different amounts and types of photoproducts formed. This additional evidence supports the idea that the "impurities" initially found on TLC and HPLC were actually photoproducts of umbelliferone. Not only would these "impurities" increase in quantity with irradiation as detected by both HPLC and TLC but were shown to convert back to a compound with an Rf value equal to that of umbelliferone on TLC. Photobiological assays were then carried out with umbelliferone and any activity could be attributed to either umbelliferone or its photoproducts and not due to an impurity.

Characterization and possible identities of umbelliferone's photoproducts

Attempts were made to characterize umbelliferone's photoproducts. As shown by TLC and HPLC, most photoproducts were more polar than umbelliferone. Photoproducts from irradiation on glass absorbed in the 254 nm range as shown by fig. 37 and those from irradiation in phosphate buffer absorbed in the 495 nm range (see fig. 39). Despite attempts to isolate and further characterize these products, reconversion and very low product yield made characterization in the realm of this study impossible. However, because umbelliferone and many other hydroxycoumarins are known to dimerize and because several anti and syn dimers are possible, it is highly possible that these photoproducts were umbelliferone dimers (Reisch and Zappel, 1992; Saigo, 1992; Shim et al., 1978; Kirkiacharian et al., 1972).

Mass spectrometry was used in an attempt to isolate the dimer but was unsuccessful because of either too little product yield or the dimer converting back into umbelliferone during the test process. In addition, because of the absorption onto a reactive surface such as silica on TLC, dimerization may have been easier than in its normal crystalline state (Dr. P. Wan, Department of Chemistry, University of Victoria, personal communication). Apparent interconversions on TLC may have been due to dimers forming and then upon photoexcitation, falling apart back to

umbelliferone (Dr. N. Towers, Department of Botany, University of British Columbia, personal communication).

Lactone-ring opening was another possibility that could have occurred but was not considered as likely by Dr. N. Towers at the Department of Botany, University of British Columbia and Dr. R. Mitchell at the Department of Chemistry, University of Victoria. Umbelliferone is known to be much more resistant to lactone ring opening in basic solutions than coumarin (Murray et al., 1982). Coumarin, when dissolved into hot dilute aqueous sodium hydroxide, hydrolyses into a solution of the lactone ring-opened product, sodium coumarinate. Acidification leads to immediate ring closure and regeneration of the coumarin structure (Murray et al., 1982). Umbelliferone in alkaline solutions however immediately forms the phenolate anion and therefore, is more resistant to hydrolysis. Much stronger treatment with alkali is required to open the lactone ring to give the products acetic acid and resorcinol (Murray et al., 1982; Fink and Koehler, 1970). Comparatively, the phosphate buffer used in this study had a pH that was too low and therefore inadequate to open the lactone ring.

Umbelliferone when irradiated with NUV has been shown to be less resistant to ring opening. Absorbance and fluorescence studies of irradiated umbelliferone with NUV in this research showed a greater breakdown of umbelliferone in higher pH's than lower ones. Fink and Koehler (1970) also showed umbelliferone breakdown in high pH when NUV irradiated and formation of an intense green fluorescence at 500 nm. With increasing irradiation at 370 nm, the fluoresence intensity at 500 nm increased and was attributed to the cinnamic acid photolysis product of umbelliferone: 2-4-dihydroxycinnamic acid. However, the pH's used in Fink and Koehler's study were much higher starting at a hydroxide concentration of 5×10^{-4} M and increasing to 5×10^{-2} M. Dosages of NUV irradiation in Fink and Koehler's study were given in minutes only making a comparison between my results and theirs impossible.

In this study, small quantities of cinnamic acids may have formed at lower pH's than those used in the studies above as greater intensities of NUV irradiation were used. As this study showed formation of products which absorbed at 495 nm, the photolysis products may have been from cinnamic acids. Mattoo (1956), Goodwin and Kavanag (1952), and Barr (1948) in Fink and Koehler (1970) have also noted that umbelliferone is photolabile at higher pH's. Wheelock (1959) and Bohme and Severin (1957) in Fink and Koehler (1970) have also previously identified the cinnamic acid product of alkaline umbelliferone photolysis.

Another possibility for photoconversion is that photoproducts could have formed from lactone ring fragmentation of umbelliferone dimers which are known to be much less resistant to ring fragmentation than umbelliferone (Saigo, 1992). Dimers may have formed from irradiation procedures on glass and TLC which with further NUV converted into ring opened products. Dimerizations and resultant openings were probably less likely in solution as umbelliferone concentrations were fairly dilute at 40 µg/ml.

Biological Activity of umbelliferone

i) Photosensitization of *E. coli B/s-1* and *E. coli B/r WP2 try⁻*

After investigation of umbelliferone's purity and photoproducts, biological testing could proceed. Umbelliferone was photobiologically active at approximately 0.1 µg/spot against *E. coli B/s-1* and 20 µg/spot against *E. coli B/r WP2 try⁻* in the TLC bioassay (see figs. 13 and 16). (0.1 µg is an approximation only and was spotted on TLC by spotting 1µl of a 1/10 dilution of a 1 µg/µl stock.) To ensure that umbelliferone was not being converted to a photoactive compound from binding to TLC, a solution of umbelliferone absorbed onto agar was tested as shown by fig. 12a and this was also found to be photoactive. Because of the differences in the amount of umbelliferone required to produce a kill zone in the bacterial strains *E. coli B/s-1* and *E. coli B/r,* the

suggested mechanism of photosensitization was likely through DNA damage as the only difference between the strains is the ability to repair DNA. Furthermore, because only the bacteria in the more concentrated area of umbelliferone were killed (see fig. 13), this compound appeared to be not very active.

This is also demonstrated in the rough and hazy border of inhibition zones in the TLC bioassay produced by umbelliferone in fig. 10a and fig. 12a as compared to those distinct zones produced by 0.5 and 1.0 µg of 8-MOP in fig. 12c against *E. coli B/s-1*. Scattered colonies could also be found growing within the photosensitization zone produced by umbelliferone that were not at all present in 8-MOP. Bacterial kill fluctuated as compared to the complete response demonstrated by 8-MOP zones.

The weak photosensitizing ability of umbelliferone was further confirmed through survival testing of *E. coli B/r WP2 try-* in solution. Umbelliferone was not very photoactive against *E. coli B/r* even at 75 µg/ml and 56.28 kJ/m^2 of NUV irradiation in phosphate buffer as shown by fig. 18. Umbelliferone/NUV treated bacteria decreased to only 62.3% of control while active furanocoumarins such as psoralen and 8-methoxypsoralen decreased bacterial survival to 0.015% and 0.8% after only 600 and 1100 Joules respectively (Ashwood-Smith et al., 1982). Photoactive cinnamic acids, cis and trans-p-methoxymethlycinnamate, produced an average of 0.7% survival after 37.5 kJ/m^2 of NUV (Ashwood-Smith et al., 1993). Pre-irradiation of the solution in sun or NUV did not increase photosensitization at all which indicated that any photoproducts were also not strong photosensitizing agents.

Oxygen is a major factor in some photoactive agents such as alpha-terthienyl which produces damage through singlet oxygen production (Downum et al., 1982). Bubbling of nitrogen or air through an umbelliferone solution had only minor and fluctuating results on bacterial survival (see fig. 19). Thus, oxygen is not a major factor in umbelliferone photosensitization. Bacterial survival

from treatment with 60 μg/ml of umbelliferone with 56.28 kJ/m^2 of NUV and air bubbling was shown to decrease to 25.6% of the control only once and could not be reproduced. Both sets of results were statistically significant on a Student's t-test to $p < 0.005$, but photosensitization could not be achieved in a classic dose/response fashion with effects plateauing at approximately 25 to 65% cell survival.

Bacterial survival curves were repeated on agar by swabbing bacteria on the surface of agar with umbelliferone incorporated into it. It was hoped that this treatment would be more effective as the light would not have to penetrate through any solution or agar barrier before reaching the target. As shown by survival curves on agar, umbelliferone was again only mildly photoactive decreasing cell survival at 40 μg/ml to 79.6% when compared to umbelliferone without NUV. Photographs of plates are presented in fig. 20. 8-MOP, in contrast, at 40 μg/ml produced 0% survival at 48.24 and 96.48 kJ/m^2 as shown by fig. 21 (70 mins. and 2 hrs.). Only after umbelliferone was increased to a concentration of 75 μg/ml and dosage increased to 96.48 kJ/m^2 of NUV (2 hrs.) was any increased kill produced. Survival decreased to a mean of 36% over 2 experiments ($p = 0.017$ and 0.008 for each). Results from 75 μg/ml are shown in figs. 21 and 22.

Standard deviations in surviving colony numbers were also extremely high in umbelliferone/+ NUV irradiated plates that were not present in others as shown by Table 3. Standard deviations for umbelliferone irradiated at 75 μg/ml and 96.48 kJ/m^2 increased 4 and 5 fold over control. These results may be due to the fact umbelliferone photosensitization was weak and that depending on gene target and particular bacterial susceptibility, DNA damage may or may not have been repairable or lethal.

Colony size appeared to be also affected as shown by fig. 22. Umbelliferone at 75 μg/ml without NUV produced colonies that were smaller than control without NUV. Furthermore, umbelliferone

plates with NUV had smaller colonies than umbelliferone plates without NUV. Umbelliferone, therefore, seemed to induce a division delay in bacteria that was increased with NUV. In the dark, umbelliferone may have intercalated into DNA altering transcription/translation or interacted with the many proteins or enzymes involved in metabolism and replication. With NUV irradiation, any cycloadducts to DNA may have required repair and alkylation of proteins may have also altered metabolism: both possibly inducing division delay (Pindur et al., 1993; Allen Smith, 1988).

Because of the survival curves in solution and agar and comparison to the activity of other established agents, umbelliferone is only a mild photosensitizing agent. Because of low reactivity, DNA damage may not have been lethal or adducts may have been easily repaired by classic excision repair mechanisms (Allen Smith, 1988). Thus, umbelliferone could not decrease bacterial survival to < 25% in both sets of experiments and results were variable between experiments and highly variable between plates of the same experiment (see Table 3 and figs. 18 and 19). A decrease in colony size in bacteria grown on umbelliferone media and scattered colonies within umbelliferone photosensitization zones on TLC bioassay also support the hypothesis that umbelliferone is a weak photosensitizing agent. Because of the differences in sensitization of the two strains of bacteria, *E. coli B/s-1* and *E. coli B/r*, demonstrated by TLC bioassay and the above data, it is likely that umbelliferone's weak photosensitizing ability is due to a combination of the low number of DNA adducts produced and of the bacteria's ability to repair them.

ii) Photosensitization of Chinese Hamster Ovary cells

Weak photosensitization may have also been the reason that CHO cells were unaffected by umbelliferone even after exposure to 160 μg/ml and 20.1 kJ/m^2 of NUV irradiation as shown by figs. 28 and 29. The number of DNA adducts may have been low and also

repairable. Also, the increased complexity and amount of membranes, proteins, histones etc. in eukaryotes may offer other targets for alkylation. Furanocoumarins have been shown to react with amino acids, proteins and lipids making it very possible that umbelliferone does as well (Midden, 1988; Murray et al., 1982). Umbelliferone is also used extensively in membrane analysis because of its amphipathic structure and small size and therefore, could interact with the many membranes in eukaryotic cells (Purohit et al., 1992; Kohen and Kohen, 1984; Sundt and Anderson, 1980). Differences in DNA secondary and tertiary structure and repair mechanisms may also make CHO cells less susceptible to cycloaddition by umbelliferone than bacteria.

In contrast, 8-MOP at 40 μg/ml produced 7% survival after only 804 Joules in this study. In Ashwood-Smith et al., (1982), 8-MOP and psoralen decreased CHO survival to 0.13% at 780 Joules/m^2 and 0.008% with 385 Joules/m^2 respectively at the same concentration. Compounds with similar structure such as cis and trans p-methoxymethlycinnamate produced an average of 5% survival at 16.2 kJ/m^2 (Ashwood-Smith et al., 1993) and 5, 7-dimethoxycoumarin produced approximately 1% survival at 9.5 kJ/m^2 (Ashwood-Smith et al., 1983). Thus, umbelliferone was tested beyond the limit at which other photoactive coumarins produce a LD_{95} and is not a photosensitizing agent in CHO cells.

iii) Frameshift mutagenesis of *E. coli lac*$^-$ ND160 and *Salmonella typhimurium* TA98

Umbelliferone does appear to interact with DNA as shown by frameshift mutagenesis of *E. coli lac*$^-$ ND160 in figs. 23 and 24. Mutagenesis was demonstrated in a dose response relationship with levels of significance at $p < 0.05$ for 60 μg/ml and $p < 0.01$ for 125 μg/ml. However, umbelliferone was inactive against *Salmonella typhimurium* TA98 in the Ames test as shown in fig. 27. This contradiction can be explained by the fact that umbelliferone was only added to the 2 mls of overlay and not to the base agar in the

test. It was observed that umbelliferone diffused throughout the base agar after 2 days of incubation and therefore, was probably not in high enough concentration to react with bacteria in overlay. *E. coli lac*$^-$ testing was repeated in an overlay method as shown by fig. 26 and umbelliferone was not shown to be active until the concentration was increased to 250 μg/ml of umbelliferone in the overlay. The Ames test was done with only 150 μg/ml of overlay. An alternate possibility could be that umbelliferone was selectively more active in *E. coli* because of sequence specificity of the target genes tested. TA98 has a frame-shift mutation in a repetitive GC sequence inactivating the histidinol dehydrogenase gene while *E. coli* has a frame-shift mutation in the *lac z* gene. Other protein, membrane and DNA structure differences affecting penetration of compound and offering other targets for interaction could also account for the difference.

Umbelliferone, however, was not a strong intercalator in *E. coli lac*$^-$ as 60 μg/ml was required to produce a significant response ($p < 0.05$) and even at 175 μg/ml, could not produce the number of mutants induced by 8-MOP at 40 μg/ml (see fig. 23). Umbelliferone increased background mutation rate by approximately 2-3 fold depending on the concentration used while 8-MOP increased it by 8-25 fold. Umbelliferone induced an average of 0.56 revertants/μg while 8-MOP induced 1.88 revertants/μg. 8-MOP results were consistent with those previously published (Ashwood-Smith, 1978; Bridges and Mottershead, 1977). 5, 7-dimethoxycoumarin, a coumarin with like structure and shown to be a strong photosensitizer, comparatively induced background mutation almost six fold at 40 μg/ml. (Ashwood-Smith et al., 1983) Spontaneous reversion rates of bacteria were within previously published parameters (Ashwood-Smith, 1978; Bridges and Mottershead, 1977; Clarke and Wade, 1975).

iv) Photobiological activity of irradiated umbelliferone

Because 5-geranoxypsoralen was shown to be inactive except through formation of photoproducts, the photoproducts of umbelliferone were also tested for photobiological activity. (Ashwood-Smith et al., 1992). Umbelliferone and its photoproducts appeared photoactive on TLC bioassay as shown by figs. 10b and c and fig. 15. Irradiated photoproducts on glass were active as well as shown by fig. 17. However, it was not initially clear whether umbelliferone was active in itself or only through formation of its photoproducts.

To determine this, umbelliferone was tested on TLC bioassay as shown by fig. 14 from 10 mins. to 160 mins. of NUV (8.04 kJ - 128.64 kJ/m^2). The inhibition zone was first evident at 10 mins. and grew in size until it reached approximately 2.5 cm in diameter at 80 mins. In contrast, fig. 15 shows 6 µg of umbelliferone irradiated on TLC for varying amounts of time. Photoproducts were first detectable at 5 mins. irradiation and quantity appeared to plateau at 18 mins. These produced only faintly discernible inhibition zones on TLC bioassay. The amount of photoproduct never exceeded 0.5 cm in diameter on TLC as well. Therefore, as photoproducts were produced in only small quantities, umbelliferone activity is probably attributed to umbelliferone and not only its photoproducts.

To confirm this in another method, 0.1, 1 and 5 µg of standard and pre-irradiated umbelliferone x 96.48 kJ/m^2 in agar were compared for photoactivity. (0.1 µg was an approximation only and was measured as per materials and methods.) As shown by fig. 16, inhibition zones were smaller in the pre-irradiated compound than in standard. These differences, however, were not statistically significant. Therefore, umbelliferone does appear to be photoactive in itself and is probably more active than its photoproducts. This is supported also by the fact that umbelliferone irradiated in sunlight for a 24 hr. period on TLC eliminated all photoactivity (see fig. 47).

Pre-irradiation of umbelliferone in phosphate buffer solution in sunlight and NUV also had no effect on *E. coli* survival. It is probable that low dosages of light produce photoproducts that are less photoactive than umbelliferone and that strong dosages of light may break down these products further eliminating all activity.

This was again demonstrated using pre-irradiated and standard umbelliferone in the frameshift test of *E. coli lac*$^-$. As shown by fig. 24, both compounds were equally active in the assay but pre-irradiated umbelliferone plates produced slightly less revertants (results not signifcant). Photoproduct conversion from irradiation on TLC was no more than 1% as estimated by eye via area and intensity of fluorescence. Thus, it is probable that both photoactivity and frameshift activity is due to either umbelliferone itself or a combination of umbelliferone and its photoproducts rather than just photoproducts themselves. If photoproducts only were active, pre-irradiation should greatly increase photosensitization and frameshift mutagenesis. As seen in fig. 10c, some photoproducts could convert back and forth into one another and back into umbelliferone. The possibility that photoproducts could be active through reversion back to umbelliferone was not excluded.

v) Inactivation of the ampicillin gene in the plasmid pTZ18R

Finally, umbelliferone reactivity with DNA was confirmed through inactivation of the ampicillin gene in pTZ18R. Transformation efficiency exhibited a 10 fold difference between the two lot numbers of cells used: 10^6 colonies/μg of DNA for lot #1 vs. 10^5 colonies/μg for lot #2 but very little fluctuation occured between cells of the same lot number. This difference was probably due to storage difficulties of lot #2 cells at -80°C which may have led to a resultant decrease in transformation efficiency. As shown by Tables 4 and 5 and transformation curves figs. 30 and 31, minor fluctuations in amount of cells used in each transformation and transformation efficiency within cells of the same lot number could

not account for the differences obtained in numbers of transformants between treated and control pTZ18R DNA.

A significant difference in number of transformants between equal amounts of DNA used in the same treatment was shown only once when comparing duplicate vials of 4.0 ng DNA treated with 8-MOP at 5 minutes. Vial A produced a 32% difference in the number of transformants when compared to vial B. In contrast, in all other experiments no signifcant difference was found between vials expected to produce equal numbers of transformants while those vials expected to produce positive results were much greater than 32%. For example, no significant difference was found between numbers of transformants for 4.0 ng control DNA with and without NUV as shown by fig. 33. Furthermore, differences in numbers of transformants produced between 4.0 ng control DNA and 0.8 ng were 79.3% ($p= 0.001$) which was much greater than the differences between vial A and B.

The difference between treated DNA with NUV and treated DNA without NUV for both coriandrin and umbelliferone was also very high ranging from 91.6% to 99.2%. The numbers of transformants also did not differ significantly for pTZ18R DNA treated with umbelliferone at 5 and 15 μg/ml and NUV irradiation and control/ NUV irradiated DNA after 24.12 kJ/m^2 ($p= 0.54$). And finally, no significant difference was found between transformants produced by control DNA without NUV, control DNA with 96.48 kJ/m^2 of NUV, coriandrin 5 μg/ml without NUV and umbelliferone 15 μg/ml without NUV as shown by fig. 35. Fluctuations between numbers in 8-MOP duplicate vials at 5 mins. NUV may have been due to experimental error. Except for this one example, this test system appeared to be a valid and reliable method of detecting DNA damage as shown by all other above examples. No significant differences were obtained by any other treatment expected to produce the same results and positive results for coriandrin or umbelliferone with and without NUV were greater than 90%. To eliminate any errors produced by experimental error or aforementioned fluctuations

between vials of the same lot number, all treatments were carried out to at least a 90% decrease in numbers of transformants for a positive result.

The coumarins, 8-MOP and coriandrin, were selected as positive controls for this test as they are both well-established strong photosensitizing agents. Also, 8-MOP is able to crosslink DNA as it has two reactive sites while coriandrin, with one reactive site, forms monoadducts only. These molecules would then shown a comparison between DNA damage caused by monoadducts plus crosslinks and monoaddducts only. Both molecules produced highly positive results with coriandrin surprisingly being much more reactive.

Umbelliferone, able to produce monoadducts only, was shown clearly as a weak photoactive agent in this test system. In sharp contrast to coriandrin which decreased transformants to 0% after only 804 Joules/m^2 at 5 µg/ml, umbelliferone treatment was increased to 48.24 kJ/m^2 of NUV and 15 µg/ml before any signifcant difference could be detected. Reaction with DNA, however, was unequivocal as a clear dose response relationship was found with either an increase in concentration of umbelliferone or NUV dose. As shown by fig. 34, pTZ18R DNA treated with umbelliferone showed a decrease from 100% of transformants without NUV to 21.6% at 1 hr NUV (48.24 kJ/m^2) at 5 µg/ml and then to 14.7% at 1 hr at 15 µg/ml. Mean percentage of transformants dropped to 4.9% at 2 hrs of NUV (96.48 kJ/m^2) at 15 µg/ml of umbelliferone as shown in fig. 34 and to 9.4% in fig. 35 ($p < 0.0005$ for both on Student's t-test when compared to control). Decreases could not be explained by NUV treatment alone as shown by control data. Therefore, pTZ18R DNA was damaged by umbelliferone plus NUV treatment as shown by inactivation of its ampicillin resistance gene. However, because the umbelliferone/DNA adduct was not isolated and characterized, the mechanism of the damage can only be speculative. As Marciani et al (1971) was able to show that umbelliferone reacted with naked DNA

in solution via cycloaddition through its C_{3-4} double bond, this mechanism is highly probable.

Our work with umbelliferone and 7-methoxycoumarin was recently confirmed in another laboratory. Mares et al (1993) also found that 7-methoxycoumarin was a photosensitizing agent which was highly effective in retarding mycelial growth and producing morphological changes in the fungi, *Microsporum cookei* as shown by transmission and scanning electron microscopy. Umbelliferone, at 100 μg/ml, induced no ultrastructural changes but retarded mycelial growth by 7%. This result was questioned for statistical significance but does suggest that umbelliferone may be a weak photoactive agent. This is the only other report that exists in the literature other than those produced by our laboratory that studies photoactivity of umbelliferone. However, as reports of antimicrobial activity of umbelliferone exist widely in the literature (see Table 1), the discovery that umbelliferone may be a weak photosensitizing agent for certain organisms may be supported.

Conclusion

In conclusion, umbelliferone was a weak photosensitizing agent against *E. coli B/s-1* and *E. coli B/r WP2 try*$^-$ but did not have any effect on CHO cells. Biological activity could be attributed to either umbelliferone or both umbelliferone and its photoproducts. Pre-irradiation of umbelliferone under NUV appeared to decrease its photoactivity in the *E. coli B/s-1* TLC bioassay while longer exposures in sunlight abolished all activity. Formation of photoproducts varied depending upon the conditions of irradiation and could not be characterized either due to low yield or reconversion back to umbelliferone. Compared to other coumarins, umbelliferone had relatively weak photoactivity. Its interaction with DNA was confirmed through frameshift mutagenesis of *E. coli lac*$^-$ ND 160 and inactivation of the ampicillin resistance gene of the plasmid, pTZ18R. However, it did not appear to frameshift

Salmonella typhimurium TA98 in the Ames test. As shown by our laboratory by Ceska et al. (1992), umbelliferone did appear to have selective photoactivity when tested with different fungii and bacteria. This selectivity of reaction requires further investigation before any explanations or exploitation can be made. Preferential DNA sequences for umbelliferone interaction and different metabolism in various organisms rendering umbelliferone inactive may be factors.

Because of the high concentrations required for frameshift or photoactivity and the high NUV doses for photosensitization, umbelliferone can be considered to be a very weak biological agent. No clinical cases of contact dermatitis have ever been reported for umbelliferone to our knowledge (Mitchell, J. and Rook, A., 1979). However, due to umbelliferone's heavy usage in research and industry and positive though weak results in frameshifting and reacting with DNA, workers should handle umbelliferone with some caution until further investigation with in vivo mammalian assays and human cell lines is done.

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APPENDIX I

FIGURES 48 - 50

Figure 48. Absorption spectrum of umbelliferone at 20 μg/ml in HPLC grade methanol on a Beckman DU - 64 spectrophotometer with scan speed set at 500 nm/min. and upper absorption limit at 2.0 units (1 cm pathlength).

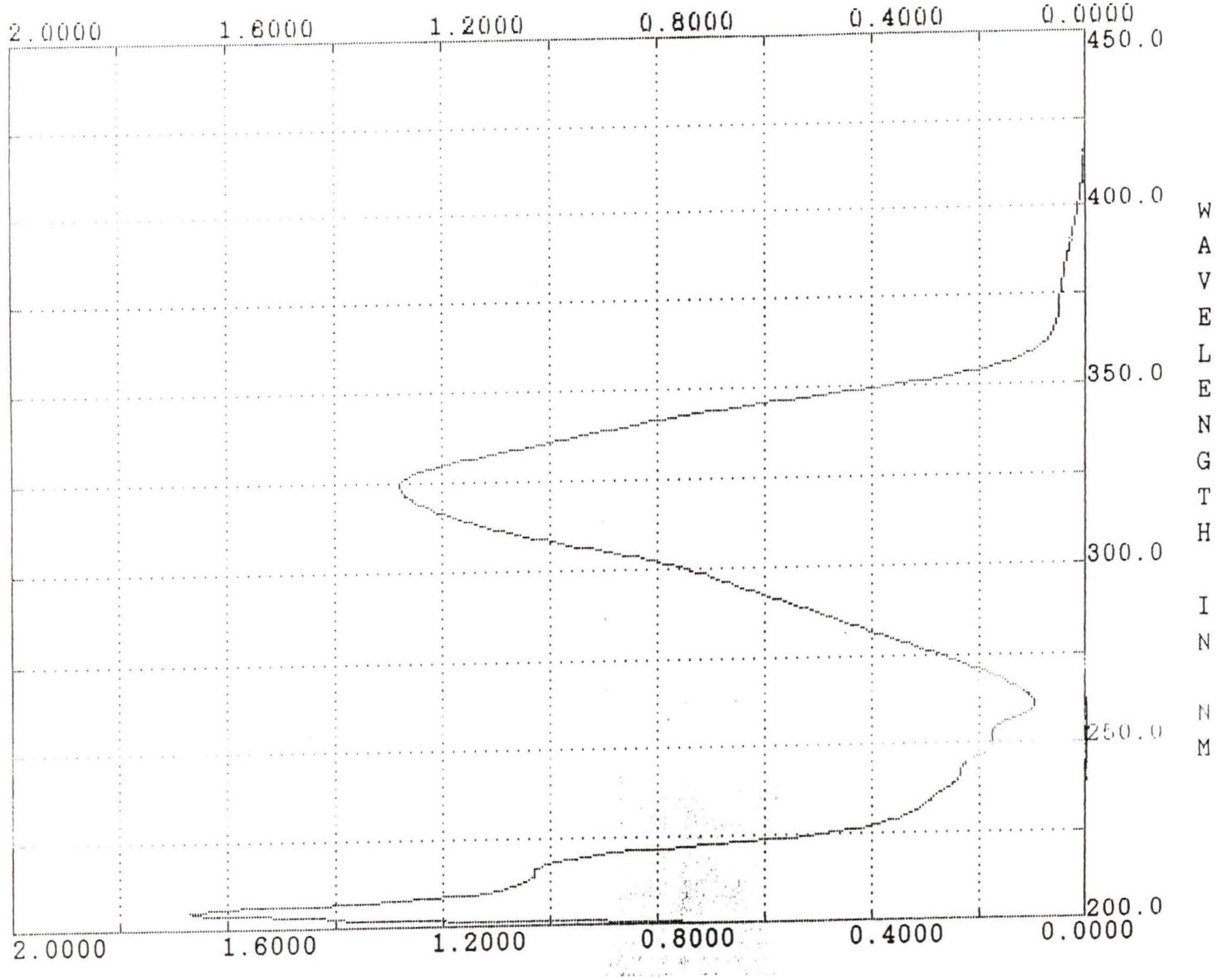

2.0000
1.6000
1.2000
0.8000
0.4000
0.0000
450.0
400.0
350.0
300.0
250.0
200.0
WAVELENGTH IN NM

Figure 49. Fluorescence spectra of umbelliferone (approximately 0.1 µg/ml: made from a 1/10 dilution of a 1 µg/ml stock) in 0.07M Sorrensen's phosphate buffer on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at different pH's: 6.0, 6.6, 7.0, 7.4, 7.8, 8.0, and 8.2 using an excitation wavelength of 330 nm and slit widths of 2/2 nm.

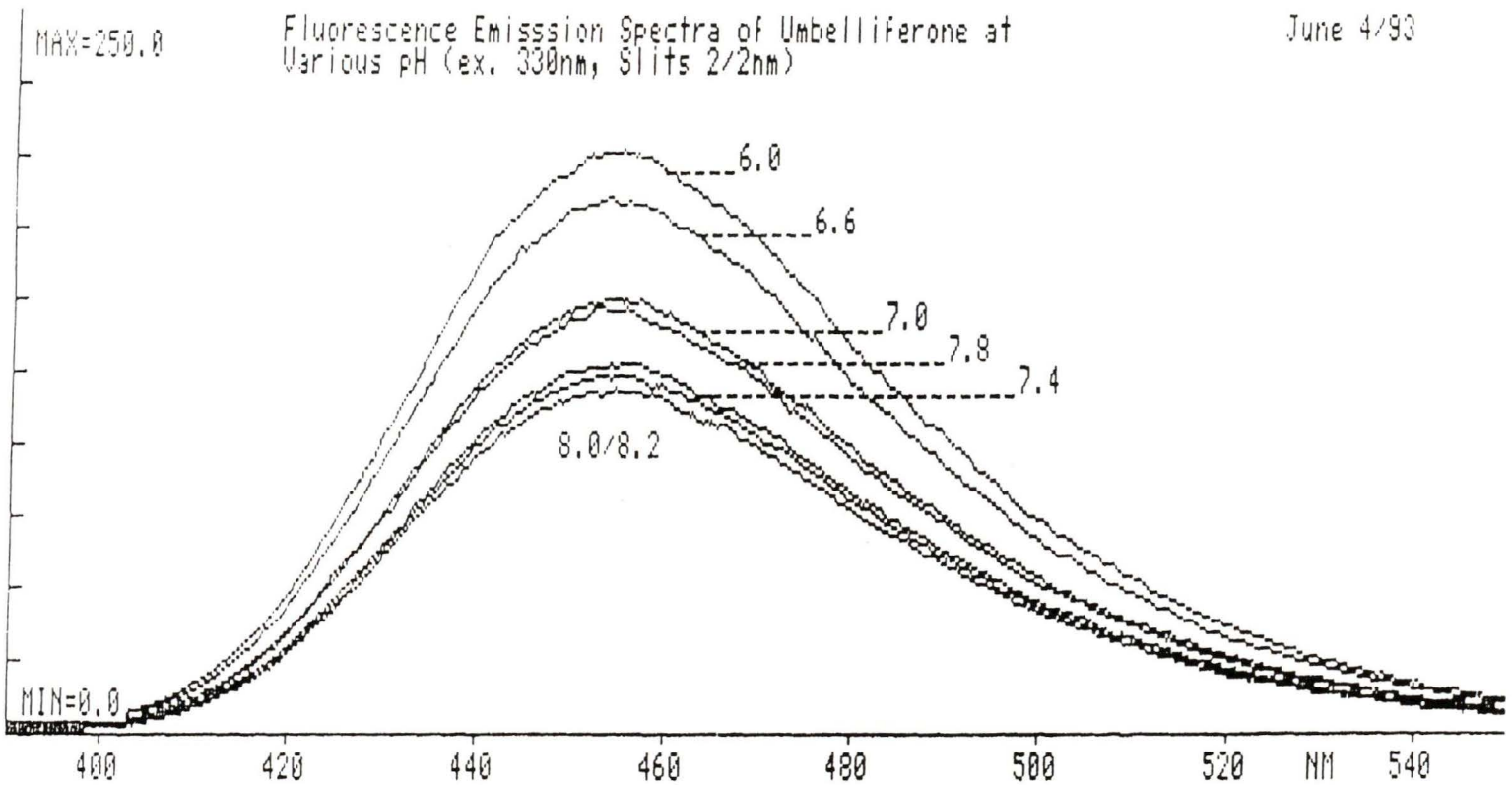
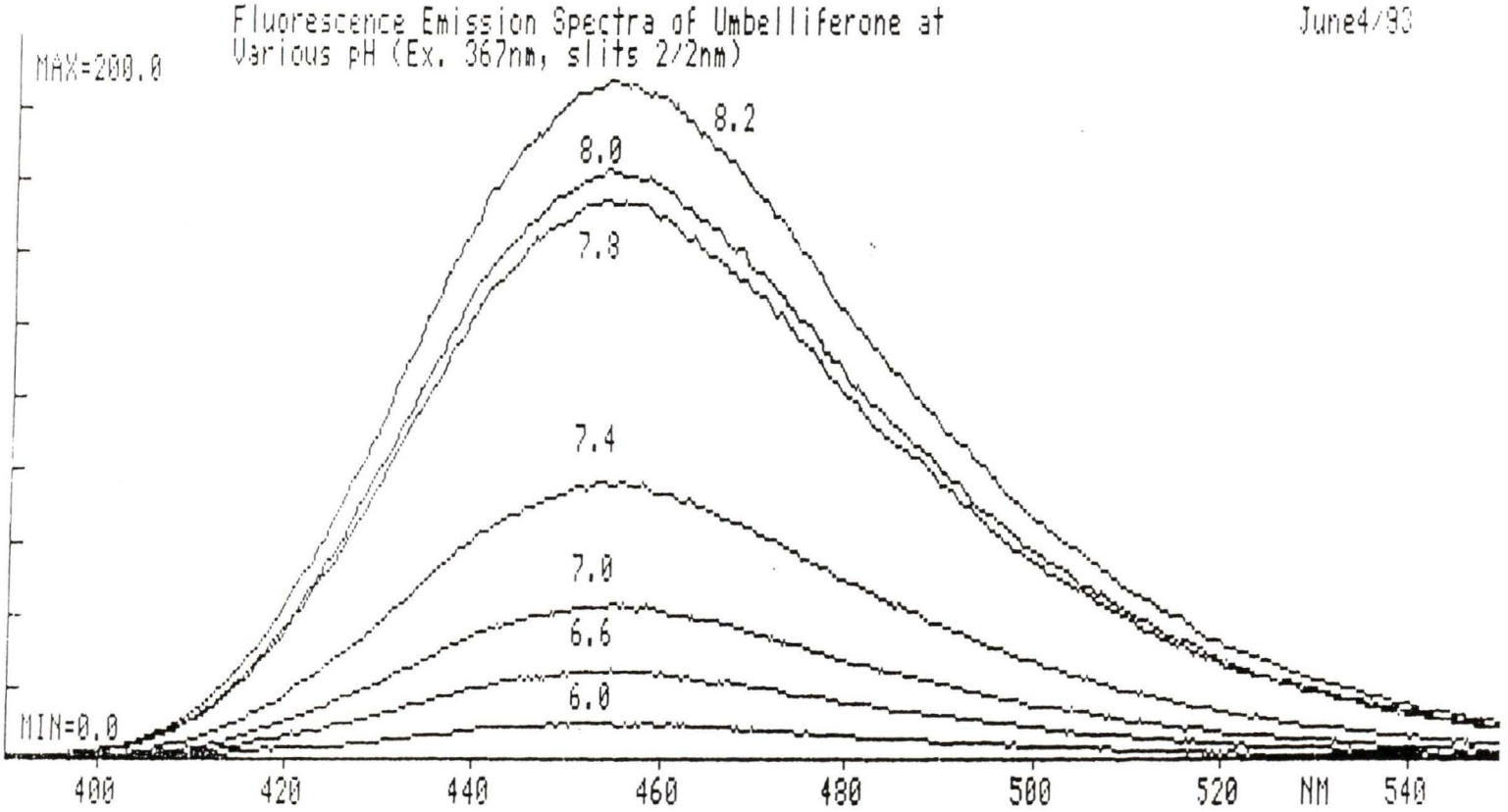

MAX=250.0
Fluorescence Emisssion Spectra of Umbelliferone at Various pH (ex. 330nm, Slits 2/2nm)
June 4/93
6.0
6.6
7.0
7.8
7.4
8.0/8.2
MIN=0.0
400
420
440
460
480
500
520
NM
540

Figure 50. Fluorescence spectra of umbelliferone (approximately 0.1 µg/ml: made from a 1/10 dilution of a 1 µg/ml stock) in 0.07M Sorrensen's phosphate buffer on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at different pH's: 6.0, 6.6, 7.0, 7.4, 7.8, 8.0, and 8.2 using an excitation wavelength of 367 nm and slit widths of 2/2 nm.


Fluorescence Emission Spectra of Umbelliferone at
Various pH (Ex. 367nm, slits 2/2nm)
June4/93
MAX=200.0
MIN=0.0
8.2
8.0
7.8
7.4
7.0
6.6
6.0
400
420
440
460
480
500
520
NM
540

APPENDIX II

REAGENT RECIPES

1) Frameshift mutagenesis of *E. coli lac*$^-$

Base agar was made as follows: 12 g of Bacto-agar in 400 ml dH_2O autoclaved, 75 ml of 2% lactose solution, 18 ml nutrient broth (Scott Laboratory), 1 ml of 250 µg/ml thiamine and 300 ml of minimal salt solution. All ingredients were filter sterilized or autoclaved. Minimal salts were made as follows: 5.8 g KH_2PO_4, 11.8 g K_2HPO_4, 0.86 g $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.15g $MgSO_4$, and 1.5 g $(NH_4)2HPO_4$ dissolved into 600 ml dH_2O and filter-sterilized.

2) Inactivation of pTZ18R ampicillin gene:

2X YT broth-- 19 g tryptone
10 g yeast extract
5 g NaCl
1 litre dH_2O mixed and autoclaved

2X YT agar-- as per above with 15 g bacto-agar

2X YT agar + ampicillin-- as per above with 50 µg/ml ampicllin
ampicillin stock 25 mg/ml filter sterilized and added to agar at time of pouring.

Nutrient agar-- as per Scott Laboratory
23 g/litre dH_2O

Lysis buffer-- 0.18 g glucose (5 mM)
10 mM EDTA (4 ml of 0.5 M stock)
25 mM Tris-HCl (5 ml of 1 M ph 8.0 stock)

dH2O up to final volume 200 ml

SDS/NaOH-- 0.8 g NaOH
10 ml of 10% Sodium Dodecyl Sulfate
dH2O up to final volume of 100 ml

Buffer III
3 M K and 5 M OAc
solution-- 60 ml 5 M potassium acetate
11.5 ml glacial acetic acid
dH2O to 100 ml final volume

QBT buffer-- 8.766 g NaCl (750 mM)
30 ml 95% ethanol (15% final [ ])
0.15% Triton X-100
QH2O up to 200 ml

QC buffer-- 1.0 M NaCl
50 mM MOPS (3- {N-Morpholino} propanesulfonic acid)
15% ethanol

QF buffer-- 1.25 M NaCl
50 mM MOPS (3- {N-Morpholino} propanesulfonic acid)
15% ethanol

APPENDIX III

REFERENCED ADDRESSES

1) Epicurian Coli SURE™ Cells are derived from the *Escherichia coli* K-12 strain. They have mutations in the following genes: *relA1, lac, recB, recJ, sbcC, umuC, uvrC*, and also contain an F' plasmid.
For further information regarding the genotype, inquiries can be made to the address below.
Stratagene
11011 North Torrey Pines Road
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Title of Thesis: Photobiological activity and frameshift mutagenesis of umbelliferone, 7-hydroxycoumarin, with bacterial and mammalian cells.

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SEPT. 24/94
(Date)