

PHOTOTOXICITY OF NEAR-UV IRRADIATED L-TRYPTOPHAN  
FOR RECOMBINATIONLESS MUTANTS OF SALMONELLA TYPHIMURIUM

by

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## INTRODUCTION

Our interest in near-UV and visible light sensitive bacterial systems is the result of a continuing study of recombinationless mutants of Salmonella typhimurium. The rec mutants so far isolated are light and UV sensitive, and no reversion of this sensitivity has been found which did not include a reversion to the rec<sup>+</sup> phenotype. This leads to the operational assumption that the light and UV sensitivity are a result of the same genetic lesion as the rec phenotype (13) (14). Therefore, an understanding of the biological damage produced by near-UV irradiation may provide direct information about the rec mutation, and ultimately insight into the regulation and functions involved in recombination. The experiments described here were initiated after the helpful communication by Dr. Robert Webb that John Lorenz observed that nutrient agar plates left in ordinary room light had a toxic effect for light sensitive Escherichia coli B phr<sup>-</sup> cells.

The experimental approach described here suggests a method for re-examination of light sensitive systems for which no chromophore has been identified. The search for a chromophore usually involves an action spectrum of the cell system, from which the chromophore is deductively determined by comparison with absorption spectra of pure substances. The damage is then characterized biologically and finally the damaged chromophore is isolated from the cell. An example of this approach to photobiology is the original work to determine the effect of UV on bacterial cells (27) (42) (51). This approach is very good for the detection of direct effects in which the cell is killed by the destruction of a necessary cell component by light energy absorbed by that component. However, if the light acts to cause indirect

damage to the cell by producing phototoxins from various small molecules which absorb light in the near-UV region, the action spectrum would produce no distinguishable peaks and no chromophore would be identified.

Many workers have made the observation that near-UV effects include growth delay, division delay, lowered respiration capacities, and threshold-type death curves. For a long time these effects were attributed to the production of peroxides and free radicals within the sensitive cell; however, it should be noted that the energy required to produce these compounds requires wavelengths a good deal shorter than 300 nm. Also, recent work with photo-sensitizers suggests that near-UV effects are probably the result of several different kinds of damage.

This set of experiments involves the basic photochemical and biological characterization of the near-UV phototoxin produced by blacklight irradiation of L-tryptophan. This investigation was undertaken to provide information about the relationship between the in vitro toxin and the in vivo light sensitivity of recA mutants of S. typhimurium.

## LITERATURE REVIEW

Comparison of UV and near-UV effects

Hollaender first characterized in a quantitative manner the effects of nonionizing UV, and near-UV light on microorganisms in 1943 (28). He observed the near-UV effects of growth delay and threshold-type death curves, and compared these with UV death curves which express nearly exponential killing. The importance of these observations was overlooked for a long time because some of the near-UV effects were attributed to small amounts of UV in the light sources used. This confusion has been recently reduced by the use of "cutoff" filters, interference filters, and improved monochromatic light sources. However, these experiments have been repeated by Epel and Krauss (18), and Jagger (34), and found to closely follow Hollaender's initial observations. The near-UV effects of growth inhibition and division delay have been attributed to light action on the electron transport system (39) and damage to the coupling of respiration and phosphorylation (37). An unexplored possibility which would fit these results would be the production of intracellular toxins by the action of near-UV light on nonessential cell parts (i.e. aromatic amino acids in the cell pool).

There is also good evidence that the effects of UV and near-UV are quite different for recombinationless (rec) mutants. It is now known that the recB<sup>+</sup> and recC<sup>+</sup> genes code for an exonuclease (47) and an endonuclease (24) respectively, but the recA lesion is still not completely characterized. It is thought that the recA gene product may be an inhibitor that regulates the B and C gene products (24). Following UV irradiation, recA mutants of S. typhimurium and E. coli rapidly degrade as much as 90% of their DNA, but recB and recC mutants do not (30). However, it has been shown that the same amount

of killing with near-UV radiation of recA mutants does not cause DNA degradation, indicating different mechanisms for far-UV and near-UV lethality (19).

Hollaender and Emmons (29) performed an action spectrum of mutagenicity which demonstrated that 260 nm was the most effective wavelength for mutagenesis, but failed to observe near-UV or visible mutagenic effects. The mutagenicity of wavelengths longer than 300 nm has recently been demonstrated by Kubitschek (41), and Webb and Malina (57) (58) and the effect characterized as oxygen dependent at wavelengths longer than 340 nm (56). The mutagenicity of wavelengths longer than 400 nm has recently been demonstrated in continuous cultures of E. coli by Webb and Malina (58). These observations suggest the possibility that near-UV and visible light act directly on the DNA, but the same observations could result from secondary effects. It is interesting to note here that although transforming factor of Hemophilus influenzae can be inactivated by near-UV radiation in vitro, the transforming ability of this DNA cannot be photoreactivated by an in vitro photoreactivation reaction. However, if H. influenzae transforming factor is UV inactivated an in vitro reaction with photoreactivation enzyme restores some of the transforming capacity of this DNA. These observations indicate that the near-UV damage to DNA is not completely the result of thymine dimerization (52), and raises the possibility of a protein DNA interaction mediated by light energy (13). Eisenstark and Ruff (16) have recently found that P22 phage which has been inactivated with near-UV is unable to reproduce on an hcr<sup>-</sup> host, but is able to infect and reproduce on an hcr<sup>+</sup> host, suggesting that near-UV damage to the phage DNA was repaired by the cells' excision repair system (16). It should be noted that in both of these cases the DNA damaged was exposed to

near-UV in the presence of significant amounts of protein, and that the observed effects could have been the result of light action on this protein component. Also, no care was taken to purify the phage, and in light of medium effects recently observed, the photoproducts may have been in the phage suspensions.

Another important difference between UV and near-UV effects is that UV damage is influenced only to a small extent by the cell growth stage (23), while near-UV is most effective for log phase cells. Finally, it should be noted in a comparison of UV and near-UV effects that there is considerable experimental data from a number of sources which indicate that UV and near-UV damage are different. It should also be noted that while UV effects are due to direct interaction of light with the cell chromosome, near-UV is a combination of several light interactions involving less essential parts of the cell.

### Photoreactivation

It is of particular interest to light sensitive problems that there are two mechanisms for photoreactivation of UV damage with near-UV light. The first type, characterized and designated as photoreactivation, involves the light-activated enzymatic cleavage of thymine dimers (52). The PR enzyme cannot be purified with retention of activity, and therefore 300 nm to 500 nm light will activate the crude enzyme to cleave dimers in vitro (60). However, a peak of activity is observed at 385 nm for bacterial PR enzyme, suggesting that *riboflavin* could be the light absorbing component (36). The second type of photoreactivation was characterized by Jagger (36) as having multiple action spectrum peaks with a main peak at 340 nm, suggesting multiple photoreceptors, and a mechanism which resembles liquid holding recovery (37) (20).

The complexity of this is increased by the recent observation of photo-recovery peaks at 313 nm, Ikenaga, Jagger, and Patrick.(31) (35) (48). An

interesting note should be made of the recent report by Brown and Webb (3) that cells damaged by 365 nm light at 0 C can be photoreactivated. It is therefore becoming clear that photoreactivation is a complex phenomenon involving several wavelengths, probably more than one photoreceptor, and possibly more than one photochemical mechanism.

#### Relation of Light Sensitive Photoreceptors to Recombination

It has been shown that the rec mutation results in sensitivity to UV, near-UV, and visible light (15). Thus an understanding of the molecular mechanism(s) of near-UV and light sensitivity of the rec system should contribute direct information about the rec lesion. There are several possible mechanisms for the light effect which could be tested. The first possibility is that the rec mutants contain an altered chromophore which is capable of absorbing the light and transferring this energy to the DNA in the same fashion as a dye in photodynamic action. Another possibility is that a function shared by recombination and replication is altered in such a way that the cell is no longer capable of recombination but can still carry out its replicative functions except when acted upon by light (13). Either of these possibilities should reveal a characteristic action spectrum with a peak activity in the region in which the chromophore described absorbs light (13).

Several observations which are important to the development of possibilities are that all three rec loci lead to near-UV and visible light sensitivity (14) (15), that recB and recC are known to be DNA nucleases (47) (24), and that recA is thought to control recB and recC gene products in wild type cells (24). This would suggest that although light sensitivity is a common denominator for the function of the rec gene products, it is very likely that

this is due to the mechanism of several phototoxins which these gene products are responsible for repairing in wild type cells. Thus, it is possible that several different photoreceptors are important for each mutant, some of which overlap and affect other mutants and some which are specific for a given lesion. If this is the case an action spectrum would not show a well defined peak, as it would require more than one wavelength to see the total effect.

### Photodynamic Action

Research dealing with the photosensitization of cells after the addition of dyes has received careful and comprehensive reviews in recent years (25) (54) (55). The photodynamic mechanism involves the light excitation of an organic photosensitizer (i.e. dyes) from the ground state to an excited singlet or triplet state, and the energy is transmitted to a nonabsorbing molecule in a reaction with oxygen (usually) with the return of the excited dye molecule to the ground state (4). There are a few anaerobic photosensitizers which have been characterized such as the furocoumarins which react with FMN (45), and DNA (46), and the psoralins (53).

Photodynamic reactions of biological interest include the photosensitized splitting of pyrimidine dimers with indole derivatives (26), which is obviously very important to photoreactivation systems involving nonenzymatic near-UV repair of dimers (33) (34) (35) (36) (49). Of special interest to this review is the methylene blue sensitized conversion of L-tryptophan to B-carboline derivatives when irradiated with 400 nm wavelength light (38). Also, there is a list of triplet state energies for organic compounds which absorb in the far-UV and the near-UV regions which allow estimations of possible reactions in vivo (27). However, the biological significance of photodynamic reactions is limited by the fact that relatively



few biological compounds which absorb light in this region have the ability to act as photosensitizers.

#### Possible Photoreceptor Molecules for Near-UV and Visible Light Effects

Several of the components of the respiratory system have been shown to be light sensitive (39). The quinones and vitamin K portions of the electron transport system were found to be light sensitive by Kashket and Brodie in 1962 (39). Epel and Butler (17) later demonstrated that cytochrome  $a_3$  can also be destroyed by light, and a number of porphyrin containing proteins have been found to be light sensitive (44). Epel and Krauss, in 1966, proposed the hypothesis that the primary damage to biological systems by near-UV and visible light was the result of a direct effect of light on these chromophores characterized in vitro. They considered the stricter oxygen requirement for near-UV and visible light effects in vivo to lend support to this idea, but if the protection observed in anoxic conditions is a result of chemical protection rather than use of anaerobic pathways, the oxygen requirement would not provide support for this hypothesis.

Mc Laren and Shugar indicate that in vitro studies of comparative enzymatic activities show that a large number of proteins can be inactivated by radiation with near-UV light (44). These effects are attributed to the photoreaction of the phenol ring of tyrosine, phenylalanine, and tryptophan, as well as cysteine in alkaline pH. The possibility of proteins as sites of photodynamic action is given special consideration in the Spikes and Livingston review (54). It is of special interest to this review that photooxidation with photosensitizers has been shown for tryptophan, tyrosine, histidine, methionine, and cysteine (54). It has also been shown that oxygen uptake with tryptophan, tyrosine, and histidine in photosensitized light reactions is associated with destruction of the aromatic ring (59).



Results in our laboratory indicate that NAD, and NADH can function as near-UV photoreceptors (Eisenstark and Yoakum, unpublished). We found that while NAD can protect recA suspensions irradiated with near-UV, NADH was found to increase the lethal effects of near-UV radiation. The possibility that these effects were the result of a light medium interaction have not been eliminated, but the fact that NAD absorbs maximally at 325 nm and NADH at 340 nm supports the possibility that they might be involved in the near-UV sensitivity of recA mutants.

It is obvious that many possible molecules for near-UV photoreactions have been described in recent years. This supports the hypothesis that near-UV sensitivity is the result of a number of wavelengths acting on several photoreceptors, and increases the probability of more than one photoreaction mechanism. However, it should be noted here that no organized effort has been reported to test for in vitro production of near-UV phototoxins using the various near-UV sensitive mutants as test organisms.

### Action Spectra

The first action spectrum was done by Gates in 1928 on the bactericidal effect of UV light (21). Gates also first demonstrated the importance of various environmental factors, and dose rate effects (22). However, monochromatic light sources were crude enough at that time to cause great difficulty in interpreting these results. Thus, Hollaender and Emmons are credited with demonstrating the mutagenicity of 260 nm UV with their action spectrum of mutagenicity (29), while Luckiesh is credited with an action spectrum of light killing which showed a peak at 260 nm UV (43).

The assumptions upon which the action spectrum is based were first organized by Loofbourow in 1948 (42). The first assumption states that the

biological effect observed is due to the photochemical inactivation of an essential cell component. The second assumption is that the quantum efficiency of this reaction is independent of the wavelength used. The third assumption says that the attenuation of the light intensity does not change the observed effect. The fourth assumption states that the relative absorbed dose of the chromophore in vivo is essentially the same as it is in vitro. The fifth assumption is that the Bunsen-Roscoe dose reciprocity law applies; i.e. the observed effect is a function of the total average incident dose and is independent of the time in which the dose is administered. These assumptions are fundamental to the application and meaning of an action spectrum (42) (44).

An interesting use of the action spectrum is the comparison of two biological phenomenon by comparing their respective action spectra. Using this method Jagger presented presumptive evidence of a correlation between growth delay and photoprotection of UV damage in E. coli (33) (37). However, it was shown that photoreactivation has several peaks, indicating that multiple photoreceptors are involved (31) (36) (49). This demonstrates the primary limitation of the action spectrum. If the effect is due to more than one event interpretation of the results is difficult as assumptions 1 and 4 are no longer valid. In fact it is becoming clear that action spectra of cell suspensions will be much less useful in determining the photoreceptors of near-UV damage than this method was for the elucidation of UV damage. This skepticism is based, in part, on the fact that peaks which are identified from these action spectra can be matched with a large assortment of near-UV absorbing molecules.

### Photochemistry

Recent developments in photochemistry are becoming of greater importance to the photobiology of the near-UV and visible regions. The technique of flash photolysis for detection of transient species in aqueous solutions during light reactions was originated by Porter in 1950 (50). This apparatus has since been refined by the use of higher energy lamps, delay switches, and spectrographic or spectrophotometric detection equipment (6) (7) (8). Duysens adapted the split beam spectrophotometer to flash photolysis equipment in 1957, and this apparatus is capable of kinetic studies of aqueous photo-reactions (12). It is of special interest to this review that Clayton has now adapted flash photolysis apparatus for fluorescence studies of aqueous reaction kinetics (9). Although the photochemistry for the light reactions of interest to a particular photobiological problem probably have not been carefully worked out, the techniques now exist to answer these questions.

For those cases in which a sizable amount of photoproduct can be made the techniques of thin layer chromatography coupled with NMR and IR studies of the separated photoproducts can be used to identify major reaction products. This approach is more applicable to photosensitized reactions (38), but can be useful in other cases also. The major drawback for non-photosensitized reactions is the quantity of product to work with is usually limited. However, if the molecules of interest were screened in advance by testing for toxicity against several different types of near-UV sensitive mutants, the photochemistry of these reactions could be worked out and the phototoxins isolated from in vivo systems.

### Significance

Photobiology is a fairly young research endeavor when compared to Radiobiology, but its significance is becoming more apparent as technology

provides more capable tools to answer questions about the biological effects of wavelengths longer than 290 nm. It has been known for quite some time that humans receive considerable amounts of near-UV light from the sun (2). Blum also demonstrated in 1945 that a thin layer of ozone in the upper atmosphere acts as a filter which removes almost all wavelengths shorter than 290 nm (2). The increasing use of sun lamps, fluorescent lighting, and blacklights (for posters, etc.) has greatly increased our near-UV exposure as a population. High-intensity blue light is currently used as a therapeutic treatment for infants with hyperbilirubinemia (1). These increase our need to know in exact terms what cellular effects are to be expected from such exposures, and the mechanism of mutagenesis observed for these wavelengths. It would also be useful to gain an understanding of the molecular events involved in repair of near-UV damage to biological systems.

It should also be noted that for the human disease Xeroderma pigmentosum, in which the skin becomes highly sensitive to sunlight (10), it has been demonstrated that these cells are defective in an initial stage of DNA repair thought to involve a nonfunctional nuclease (9) (10). This disease could very well be a near-UV effect as recent observations indicate that near-UV light can damage DNA. This idea is supported by the fact that very little 260 nm UV radiation reaches the earth while considerable amounts of near-UV does reach the earth (2).

## MATERIALS AND METHODS

Materials

## i) Bacterial Strains and Phage

S. typhimurium LT2 derivatives, used in all toxicity experiments, are listed in Table 1. E. coli B/r/t<sup>-</sup>, and T5 phage grown on E. coli B/r were used for the mutagenicity test.

## ii) Media

M9 medium was prepared by mixing 1 part M9 salts (10X concentrated) with 9 parts distilled water supplemented with 1% of 40% glucose, 10% of 12 g/liter  $\text{MgSO}_4$  and 10% of 1.47 g/liter  $\text{CaCl}_2$ . 10X M9 salts contain the following (g/liter):  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 132.3;  $\text{KH}_2\text{PO}_4$ , 30;  $\text{NH}_4\text{Cl}$ , 10; and  $\text{NaCl}$ , 5.

M9 medium for nephlostast continuous culture was made the same as M9 medium but supplemented with the following (g/liter): glucose, 150; L-tryptophan, 2; and thymine, 2.

Stock A, for addition to irradiated broth samples, contains the following (mg/liter): L-tryptophan, 160; L-lysine, 160; L-histidine, 160; and L-leucine, 160. Stock A was filter sterilized and stored at 4 C.

Stock B was a filter sterilized solution of 20X M9 salts containing the following (g/liter):  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 264.6;  $\text{KH}_2\text{PO}_4$ , 60;  $\text{NH}_4\text{Cl}$ , 20;  $\text{NaCl}$ , 10;  $\text{NH}_4\text{Cl}$ , 20;  $\text{MgSO}_4$ , 24; and  $\text{CaCl}_2$ , 2.94. Stock B was stored at room temperature.

Stock C was a solution of 10X M9 salts which contained the following (g/liter):  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 132.3;  $\text{KH}_2\text{PO}_4$ , 30;  $\text{NH}_4\text{Cl}$ , 10;  $\text{NaCl}$ , 5;  $\text{MgSO}_4$ , 12; and  $\text{CaCl}_2$ , 1.47. Stock C was supplemented with 10% of 40% glucose and 10% of 200 mg/liter thymine. This solution was filter sterilized, stored at 4 C, and warmed with stirring before use.

Stock D was prepared by mixing 8 parts of stock C with 2 parts of distilled water.

Table 1. Bacterial Strains of S. typhimurium used to Test Phototoxicity

KSU Strain #	Genetic Markers	Phenotypic Characteristics
1619	lys	lysine 20 ug/ml
3916	trp thy <u>recA</u>	UV and light sensitive, tryptophan 20 ug/ml, thymine 1 ug/ml
2480	trp <u>recA</u>	UV and light sensitive, tryptophan 20 ug/ml
3595	his <u>recA</u>	UV and light sensitive, histidine 20 ug/ml
3593	leu <u>recA</u>	UV and light sensitive, leucine 20 ug/ml

2X minimal agar contains 18 g of Ionagar in one liter of distilled water.

Nutrient broth was prepared by mixing 8 g of Ionagar, 8 g of dehydrated nutrient broth and 5 g of NaCl in one liter of distilled water.

Nutrient agar was prepared by mixing 13.5 g of Ionagar, 8 g of dehydrated nutrient broth and 5 g of NaCl in one liter of distilled water.

Nutrient overlay agar contains the following ingredients (g/liter): dehydrated nutrient broth, 8; NaCl, 5; and Ionagar, 6.

### iii) Chemicals

Purified cyclohexanone (Baker and Adamson Allied Chemical Corp.) was used in extraction procedures. Grade A L-tryptophan was purchased from Calbiochem. Purified nitrogen (99.997% pure) was purchased from Matheson.

### iv) Light Sources and Filters

A 15 watt GE germicidal UV light, producing mostly 253.7 nm UV light, was used for all UV sensitivity tests.

A pair of integral filter 15 watt GE F15T8 • BLB blacklights, at a distance of 4 cm. from the sample producing  $150 \text{ erg/mm}^2/\text{sec}$  light energy, was used for all blacklight irradiations. This light source produces energy between 290 nm and 450 nm with a peak of energy at 365 nm wavelength (32).

Monochromatic light was provided by a xenon arc lamp in a Schoeffel housing coupled with a Bausch and Lomb 500 mm monochromator. The units of the system consisted of: a Bausch and Lomb 500 mm monochromator; a Schoeffel lamp housing LH152N; a Hanovia 975c 2500 watt xenon arc lamp; Schoeffel LPS400 power supply; a Schoeffel LPS400S 200 ampere direct current starter; and a predispersion prism. The light energy was measured with a YSI Kettering radiometer.

Corning glass "cutoff" filters were used at wavelengths longer than 310 nm to eliminate short wavelength scattered light. The %T spectrums of the filters are listed in Table 2.

### Methods

#### i) Cell Growth

Liquid cultures were grown with aeration from single colonies at 37 C. Growth was followed by optical density at 600 nm in a Klett-Summerson photo-electric colorimeter and the culture was used at mid-log phase.

Nephlostad continuous cultures were used to provide log phase cells for the mutagenicity and action spectrum experiments. The Nephlostad regulates the number of cells in a culture by monitoring the density of the culture and adding an aliquot of medium when the density reaches a pre-set value. This method of density regulation eliminates many of the problems associated with continuous culture techniques.

Table 2. Corning Glass Cutoff Filters used to Decrease Scattered Light

Filter #	$\lambda$ (nm)	%T Spectrum 1.0%T limit (nm)	%T at $\lambda$ used
0-53	310	290---	55%
0-54	320	305---	60%
7-51	340	320-405	60%
7-51	350	320-405	68%
0-52	360	350---	45%
0-52	370	350---	68%

## Explanation

The filter # is the catalogue designation for each filter and  $\lambda$  is the wavelength setting of the light source during the radiation. The %T spectrum limits reflect the transmittance limits for each filter, and %T at  $\lambda$  is the transmittance of the peak energy at each wavelength setting.

## ii) Marker Stability Test

Immediately before use of the culture, cells (ca.  $2 \times 10^4$ ) were spread on two nutrient agar plates. One plate had a cross of white tape on the back to block light, so that it would contain a dark control region. This plate was inverted and blacklight irradiated for 60 minutes. Half of the second plate was exposed to UV ( $100 \text{ erg/mm}^2$ ) and both plates were incubated overnight at 37 C. Failure to form colonies in the exposed areas of both plates was considered positive evidence for the retention of the recA mutation. The covered areas provided a control and growth of unexposed cells served as a distinct visible contrast.



### iii) Medium Photoreceptor Test

Each medium and medium ingredient listed in Table 3 was irradiated with blacklight for 180 minutes then assayed for toxicity using all of the strains listed in Table 1. A 6.0 ml aliquot of each was placed in a 100mm petri dish and 1.0 ml of Stock A and 1.0 ml of Stock D was added to each sample. Each dish was then seeded with 0.1 ml (ca.  $1 \times 10^3$ ) log phase cells and incubated at room temperature for 30 minutes. 2X minimal agar was prepared by adding 20 ml of Stock B to 380 ml of agar just prior to use. The dishes were then solidified by adding 8.0 ml of prepared 2X minimal agar at 48 C to each dish. The plates were then incubated overnight at 37 C.

### iv) Testing Stability of Phototoxin

A 30 ml solution of L-tryptophan was irradiated with blacklight for 200 minutes, and 10 ml of this was stored at 4 C while 20 ml was placed in a dialysis bag and dried overnight at room temperature with a fan. The residue was then dissolved in 20 ml of distilled water and separated into two 9.0 ml aliquots. 1.0 ml of 2.5N HCl was added to one and 1.0 ml 2.5N NaOH to the other and both were hydrolyzed at 90 C for 60 minutes in a heating block. Following hydrolysis, the samples were cooled in ice and neutralized to pH 7.0 then assayed for toxicity using the method described above with KSU 3916 as the test organism.

### v) Amino Acid Analysis of Irradiated L-tryptophan

A 204.2 ug/ml solution of L-tryptophan was exposed to blacklight and a sample removed every 30 minutes for 240 minutes. The samples were then applied to the acidic, basic and neutral columns of a Beckman 120C Amino Acid Analyzer. The peaks recorded were compared to standards prepared from unirradiated sample. Separate standards were prepared to identify trace peaks.

vi) Extraction of Toxicity with Cyclohexanone

Two 50 ml aliquots of L-tryptophan (10 mg/ml) were irradiated with blacklight for 240 minutes. A portion of this was stored at 4 C and 80 ml was extracted at room temperature by shaking with cyclohexanone. An unirradiated sample was extracted and treated similarly to provide a non-toxic control for contrast.

The phases were separated by low speed centrifugation for 10 minutes and each fraction was placed in a round bottom flask. The water and cyclohexanone fractions were evaporated at 37 C and 55 C respectively with a 0.01 atmosphere vacuum. The residues were washed 3 times with ether and dried at room temperature with a 0.01 atmosphere vacuum; then the residues were dissolved in ethanol and water was added until the concentration was 50%. Finally, the alcohol was removed by evaporation at 45 C in 0.01 atmosphere vacuum until 1 minute after the boiling stopped. The samples were then serially diluted to 1/32 and the toxicity assayed using KSU 3916 as the test strain. The appropriate dilution of Stock C was added to each tube and 0.1 ml of log phase cells (ca.  $1 \times 10^5$ ) was added to each tube. The samples were then incubated for 30 minutes at 37 C with shaking, diluted and spread on nutrient agar plates and the survivors were scored by ability to form colonies after 24 hours incubation.

vii) Fluorometry of Cyclohexanone Extracted Photoproduct

A solution of L-tryptophan (10 mg/ml) was irradiated with blacklight and extracted with cyclohexanone. A non-irradiated tryptophan solution was similarly extracted and the two extracts scanned at 10 nm intervals for fluorescence with an Aminco Bowman photofluoremeter.

viii) Test for Oxygen Requirement of the Photoreaction of L-tryptophan

A solution of L-tryptophan (10 mg/ml) was divided into two samples and placed in pyrex cuvettes with bubble arms attached; a cork with a syringe needle thru it was placed in the top of each cuvette to limit the size of the gas exit and minimize "contamination". One sample was purged with air from an aquarium pump while the other was purged with purified nitrogen for 30 minutes prior to light exposure. The cuvettes were sterilized just before the experiment with a Penray UV light and the gasses were filtered with sterilized cotton filters. At time zero the blacklight was turned on and 0.5 ml were removed every 30 minutes for 210 minutes and stored overnight at 4 C.

The samples were warmed to 37 C and 0.05 ml of Stock C were added to each tube. The test strain was KSU 3916 from a nephlostad continuous culture added in 0.1 ml aliquots (ca.  $2 \times 10^4$  cells) to each sample. The samples were incubated with shaking for 30 minutes at 37 C; then spread on nutrient agar plates and the survivors counted after 24 hours incubation.

ix) Standardization of Incubation Time for Maximum Toxicity

A solution of L-tryptophan (10 mg/ml) was exposed to blacklight and a 15 ml sample removed at time zero, 30, 60, 120, and 180 minutes. The samples were stored overnight at 4 C and divided into six 2.5 ml samples, and the samples were assayed the following day.

The tubes were warmed to 37 C and 0.25 ml of Stock C were added to each sample. Then 0.1 ml of KSU 3916 (ca.  $2 \times 10^4$  cells) from nephlostad continuous culture were added to each sample. The tubes were placed on a shaker in a 37 C room and sampled by spreading on nutrient agar plates in quadruplicate at time zero, 10, 20, 30, 45, and 60 minutes incubation with the phototoxin. Survival was determined by the ability to form colonies after 24 hours incubation on nutrient agar plates.

#### x) Action Spectrum

The action spectrum was accrued at 10 nm wavelength intervals, using a Schoeffel xenon arc lamp coupled with a Bausch and Lomb defraction grating monochromator with a predispersion prism. The monochromator entrance and exit slits were set at 6 nm which emits a 10 nm wavelength band with a peak at the monochromator setting and emission limits of 5 nm to each side of the peak. It should be noted that a small amount of heterogeneous scattered light was present during all unfiltered irradiations which could have contributed to the photoreaction if the reaction required more than one wavelength. Corning glass "cutoff" filters were used for wavelengths longer than 310 nm to eliminate short wavelength scattered light from the monochromator. The same quartz cuvette with bubbling arm attached was used for all irradiations, and the cuvette was sterilized prior to each exposure with a Penray UV lamp.

The  $I_0$  was measured at the front of the cuvette with a USI Kettering radiometer before each irradiation. The light energy was also measured at the back of the cuvette empty and filled with sample to provide a %T value for each irradiation. The solution was then bubbled with air and 0.5 ml samples were removed at 10 or 30 minute intervals and stored overnight at 4 C. Samples were warmed to 37 C and 0.05 ml of Stock C were added to each sample, then 0.1 ml of KSU 3916 (ca.  $3 \times 10^4$  cells) from a nephlostast continuous culture were added to each tube. The samples were then incubated at 37 C for 30 minutes and plated in quadruplicate on nutrient agar. Survival was measured by the ability to form colonies after 24 hours incubation on nutrient agar plates.

The reciprocity of the dose-concentration relationship was checked at 280 nm and 320 nm by irradiating several concentrations of tryptophan (mg/ml):

0.05; 0.025; 0.01; and 10.0; 5.0; and 2.0 respectively. Each of these concentrations were sampled at time zero, 30, 60, and 150 minutes, and assayed for relative toxicity as described above.

xi) Test of Irradiated L-tryptophan for Mutagenicity

E. coli B/r/t was grown, and exposed to the suspected mutagen, in a nephlostast continuous culture. The cells were grown for 28 hours, with eight generations of limitation, before exposure to the irradiated tryptophan.

L-tryptophan (10 mg/ml) was irradiated with blacklight for 200 minutes, and filter sterilized just prior to the experiment. At time zero 100 ml of this solution were added to approximately 2 liters of M9 medium in the medium reservoir, resulting in a final concentration of 500 mg/liter of irradiated tryptophan. The suspected mutagen was then added to the culture gradually as medium additions were made. The Nephlostast monitored and recorded the density of the culture every 60 seconds, and when the density exceeded a pre-set value medium addition was triggered and the culture diluted.

The population was tested for T5 resistant mutants by collecting the overflow of the culture for 10 minutes in a tube, cooled to 4 C by an ice bath, at times zero and various times after the addition of the suspected mutagen for 50 hours. The samples were diluted and plated on nutrient agar plates in quadruplicate to determine the number of viable cells, and T5 resistant mutants were scored by placing 0.1 ml of the sample on four iron agar plates and adding 0.2 ml of T5 phage to each drop and spreading the mixture on the plate. The plates were incubated at 37 C and survivors scored by their ability to form colonies in 24 hours.

## EXPERIMENTAL RESULTS AND DISCUSSION

## i) Medium Photoreceptor Test

Table 3 shows that L-tryptophan is the only medium ingredient tested which becomes toxic after irradiation. Also, the observed toxicity is 1000 fold more effective for recA mutants than wildtype cells, and recA mutants which do not require an exogenous source of tryptophan are no less sensitive to the phototoxin than recA mutants which do require tryptophan. Finally, Table 3 indicates that the toxic effect observed for recA mutants is great enough to provide a sensitive system with which to study this phenomenon.

The results of this experiment do not eliminate any of the molecules tested as candidates for medium, or cell photoreceptors. An experiment to do this would require an exhaustive number of test solutions which contained all possible combinations of these molecules. It should be noted that the requirements for detection by this method are quite strict, and many possible photoreactions are overlooked. Those photoreactions which require trace amounts of catalyzing substances, and those involving interactions between multiple substrates, would not be detected. However, this point only emphasizes that the expansion of this technique to several hundred reaction mixtures using an appropriate set of near-UV sensitive mutants as test organisms would be expected to identify several interesting phototoxins for further study.

## ii) Test of Stability of Phototoxin

Table 4 demonstrates that the phototoxin is stable to strong oxidative hydrolysis, but is completely destroyed by reductive hydrolysis. It is also shown that while drying resulted in a 38.7 percent loss in toxicity, subsequent basic hydrolysis at 90 C caused no greater decrease in toxicity.

Table 3. The Relative Phototoxicity of Irradiated Medium Ingredients  
(% Survival)

Medium Ingredient	Concentration Irradiated	KSU Strains Tested				
		1619	2480	3916	3595	3593
Nutrient Broth	1X	100	3.4	0	6.8	5.4
M9 + lys, trp, his leu, thy	1X	97	30	12	25	29
L-tryptophan	20,000 ug/ml	6.5	0	0	0	0
L-tryptophan	200 ug/ml	84	0	0	0	0
L-tryptophan	20 ug/ml	99	32	9.3	33	21
L-tyrosine	200 ug/ml	87	99	111	94	98
L-lysine	200 ug/ml	99	89	96	88	94
L-histidine	200 ug/ml	110	99	103	102	112
L-leucine	200 ug/ml	95	107	106	100	99
L-proline	200 ug/ml	99	95	100	95	97
L-cysteine	200 ug/ml	97	92	98	108	90
Glucose	1%	93	94	89	99	109
Distilled H <sub>2</sub> O	---	111	92	88	100	101

Also, the observed loss of 39 percent toxicity after the drying step may have been due to handling losses and differences in solubility.

The stability of the phototoxin eliminates the possibility that the observed effect could be the result of a peroxide, free radical, polymer, or other highly labile substance. The observation that acid hydrolysis destroys the toxic effect while basic hydrolysis does not, could indicate that the tryptophan structure is maintained,(and) or that the toxicity is the result of a strongly oxidative functional group.

### iii) Amino Acid Analysis of Irradiated L-tryptophan

Figure 1a is the  $\Delta$ -aminovaleric acid and L-tryptophan standards prepared for comparison with irradiated L-tryptophan. Figure 1b is a sample of the L-tryptophan solution prior to blacklight radiation, and Figure 1c shows the peaks found after 240 minutes light exposure of this solution. Comparison of Figures 1b and 1c indicate that the reaction resulted in a 92 percent decrease in L-tryptophan in the solution with the appearance of a trace of  $\Delta$ -aminovaleric acid and a sizable quantity of ammonia. This indicates that the photoreaction results in a deamination of the L-tryptophan, or some primary photoproduct.

Since the amino acid analyzer uses ninhydrin reagent for the detection of amino acids, and this reagent is specific for  $-\text{NH}_2$  or free  $-\text{NH}_3$  groups; it is reasonable to assume that the  $\text{NH}_3$  produced is the result of deamination of the  $\alpha$ - $\text{NH}_2$  group of the alanine side chain. The  $\Delta$ -aminovaleric acid peak could indicate that cleavage of the pyrroline ring of the L-tryptophan has occurred during the reaction as this is a five carbon molecule. However, it is possible that a reaction took place between two alanine side chains resulting in the five carbon  $\Delta$ -amino acid without ring cleavage.

Table 4. Stability of Photoproduct

Conditions	3916 % Survival	% Toxicity Lost
Stored at 4 C	2.8	----
Drying	42	39
0.25N HCl at 90 C	94	91
0.25N NaOH at 90 C	41	38





### Explanation

Figures 1a - 1c are recordings of the peaks observed for the amino acids by reaction of the column elutants with ninhydrin and reading the OD of the reactant at three wavelength settings spectrophotometricly. Figure 1a is an assay of the L-tryptophan solution before irradiation, Figure 1b is a standard prepared by application of a solution with 0.15  $\mu\text{M}/\text{ml}$  of aminovaleric acid and L-tryptophan, and Figure 1c is an assay of the L-tryptophan solution after 240 minutes irradiation.

**THIS BOOK  
CONTAINS  
NUMEROUS PAGES  
WITH DIAGRAMS  
THAT ARE CROOKED  
COMPARED TO THE  
REST OF THE  
INFORMATION ON  
THE PAGE.**

**THIS IS AS  
RECEIVED FROM  
CUSTOMER.**

Figure 1a

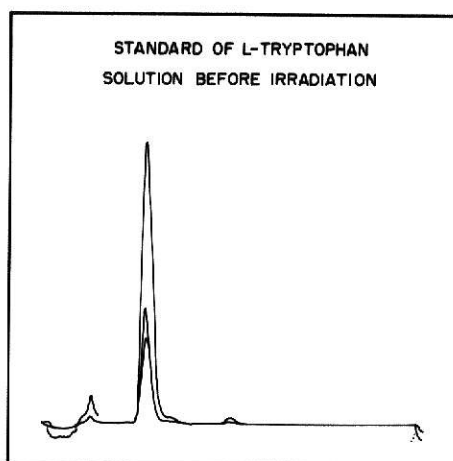


Figure 1b

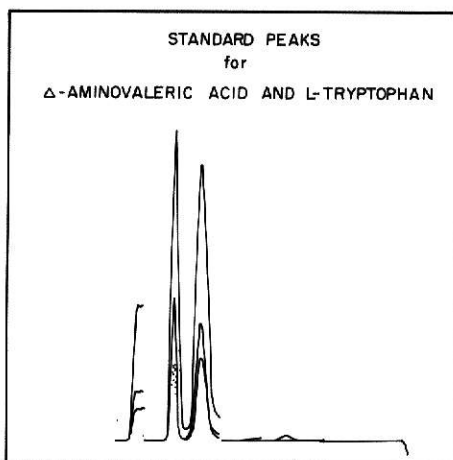
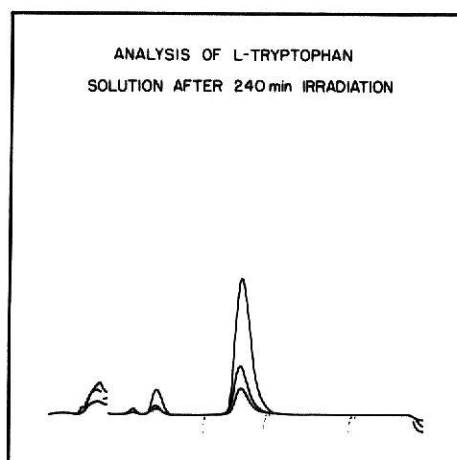


Figure 1c



#### iv) Cyclohexanone Extraction of Toxicity

Comparison of the percent survival values listed in Table 5 indicates that the extraction procedure leaves solvent residues in the samples which account for a fraction of the observed toxicity. However, comparison of the irradiated samples with controls demonstrates that this could only account for 0.24 percent of the toxicity of the cyclohexanone extract of irradiated tryptophan, and 22 percent of the toxicity remaining in the irradiated solution after cyclohexanone extraction ( $H_2O$  fraction, irradiated). Table 5 also shows that while the toxicity observed for the cyclohexanone extract was nearly the same as that of the unextracted irradiated solution, the toxicity of the extracted solution was decreased 40-fold. This indicates that the phototoxin is more soluble in cyclohexanone than water.

This solubility in cyclohexanone was demonstrated after testing several organic solvents, including cyclohexane, with negative results. The major difference in these solvents was the unique association of a polar functional group (O) with a non-polar, saturated ring. The solubility of the phototoxin in cyclohexanone suggests that it may be of intermediate polarity and could have a similar functional group. This preferential solubility also provides a method for partial purification of the phototoxin, as tryptophan is only 0.3 percent soluble in cyclohexanone.

#### v) Fluorescence of a Photoproduct in Cyclohexanone

Figure 2a shows that a fluorescence scan of the cyclohexanone fraction of extracted irradiated tryptophan contains a photoproduct which is excited by 340 nm light to emit 400 nm light. Figure 2b shows that the cyclohexanone fraction of extracted unirradiated tryptophan contains no fluorescing substance

Table 5. The Relative Toxicity of Extracted Fractions of Irradiated L-tryptophan (% Survival) as Compared to Non-Irradiated L-tryptophan Extracted Fractions

Dilution	Irradiated L-tryptophan Not Extracted	Irradiated L-tryptophan Cyclohexanone	Irradiated L-tryptophan H <sub>2</sub> O Fraction	Non-Irradiated L-tryptophan Cyclohexanone	Non-Irradiated L-tryptophan H <sub>2</sub> O Fraction	Irradiated Distilled H <sub>2</sub> O
1/1	.25	.13	12	54	48	91
1/2	.61	.19	19	80	74	111
1/4	.75	.35	35	101	78	113
1/8	2.	.66	52	84	101	90
1/16	15.	2.	70	86	96	109
1/32	28.	6.	74	87	104	83



### Explanation

Figures 2a and 2b are recordings of light energy amplified by a phototube to measure small amounts of fluorescent light produced by light excited molecules in the solution assayed. To measure fluorescence the emission wavelength was constant and the excitation wavelength was scanned, if a peak was recorded it was labeled with the value held constant. Thus, the first part of Figure 2a shows that the peak emission wavelength for extract of irradiated L-tryptophan is 400 nm, and the second part of Figure 2a shows that the excitation peak for irradiated L-tryptophan is 360 nm. Figure 2b shows that unirradiated L-tryptophan did not fluoresce.



Figure 2a

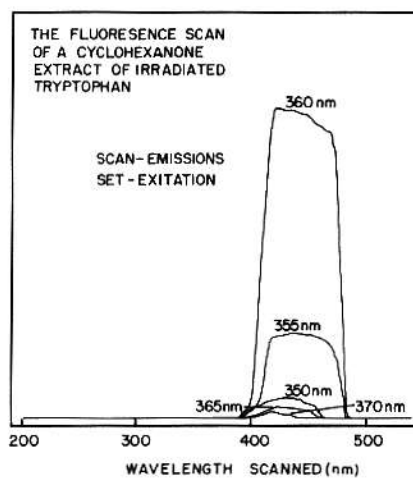
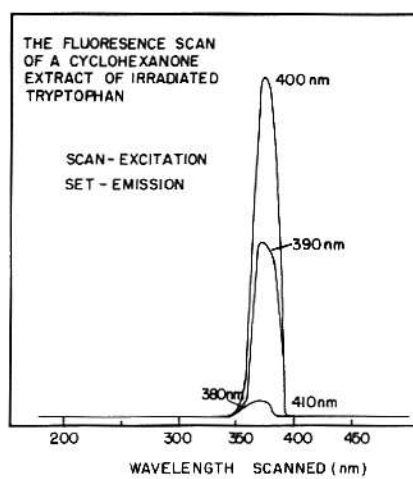
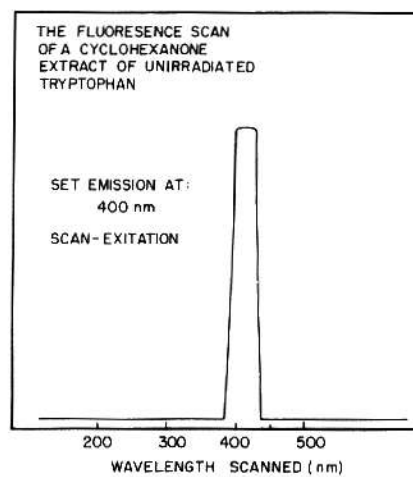


Figure 2b



even when scanned at 10,000 fold greater sensitivity. This indicates that the fluorescence observed for the cyclohexanone extract of irradiated tryptophan is due to a product of the light reaction.

The observed fluorescence could be a property of the phototoxin, or it could be the result of some nontoxic by-product of the photoreaction. Although fluorescence properties contribute little knowledge about molecular structure, the kinetics of the reaction could be studied using flash photolysis with fluorescence detection (60). This would also provide the means with which to do an action spectrum from which the quantum yield could be calculated, and group absorption or biphotonic effects accurately demonstrated. This property could also be used to develop a method to screen in vivo systems for this photoproduct, and demonstrate directly the importance of this photoreaction to the sensitive cell system.

vi) Test for Oxygen Requirement in the Photoreaction of L-tryptophan

It is shown in Figure 3 that the air purged solution of tryptophan produced 100 times more phototoxin than the  $N_2$  purged solution through both samples were exposed to the same dose of blacklight in identical conditions. This result strongly suggests that the action of blacklight on tryptophan which produces a phototoxin requires oxygen, meaning that the reaction of interest is a photooxidation.

However, it should be noted that air is a mixture of gases consisting principally of  $N_2$ ,  $CO_2$ ,  $O_2$ , and traces of the inert gases. The most likely possibilities in this mixture are  $O_2$  and  $CO_2$ , since  $N_2$  prohibited the photoreaction. Molecular oxygen is much more reactive than  $CO_2$ , thus it seems reasonable to conclude that the reaction is a photooxidation. Assuming that the reaction mechanism is a photooxidation raises the interesting possibility that in vivo protection observed with anoxic conditions could be

due to inhibition of the photoreaction rather than the metabolic pathway which the cell is using during radiation (13).

vii) Standardization of Incubation Time for Maximum Toxicity

The results in Figure 4 show, that regardless of the amount of toxin present in a given sample, its toxicity for a population of test cells is fully expressed within a 45-minute incubation period. Though the maximum incubation required is 45 minutes, it is indicated that more toxic samples, those which received greater doses of blacklight, expressed their toxicity in less time than samples of lower toxicity. Solutions that received light doses less effective than 30 minutes of blacklight exposure were considered undetectable in this system, and a standard incubation period of 30 minutes was considered sufficient for the expression of relative toxicity.

The observation that toxicity is expressed within a given time interval suggests that the toxic effect could be specific for a phase of the bacterial growth cycle, and that cells in, or about to enter, this "sensitive phase" are killed by the toxin while the rest of the population experiences sublethal effects. However, these results could also be explained by the fact that all incubations were carried out in the region of "cell excess", in which case a similar set of curves would be expected.

viii) Action Spectrum of Toxicity of Irradiated L-tryptophan

Figure 5 is a plot of "Action" in  $\text{mm}^2 \text{erg}^{-1}$ , a quasi-rate constant of the phototoxic reaction, versus wavelength of light used to irradiate L-tryptophan. The efficiency of a given wavelength to produce phototoxin was determined from the time of irradiation required to kill 50 percent of a test cell population as extrapolated from the graphs in Figures 6a thru 6l. The  $\text{LD}_{50}$  dose taken from these graphs was then corrected to the Average Incident  $\text{LD}_{50}$  Dose for each wavelength by application of a correction factor (32) taken from a graph



### Explanation of Figure 3

The points represented are the averages of four platings for each sample. The open circles  $\bigcirc$  represent the number of KSU 3916 test cells which survived the prescribed incubation with L-tryptophan irradiated the length of time indicated at each point while being purged with  $N_2$ . The closed triangles  $\blacktriangle$  represent similar points for a solution of L-tryptophan purged with air.

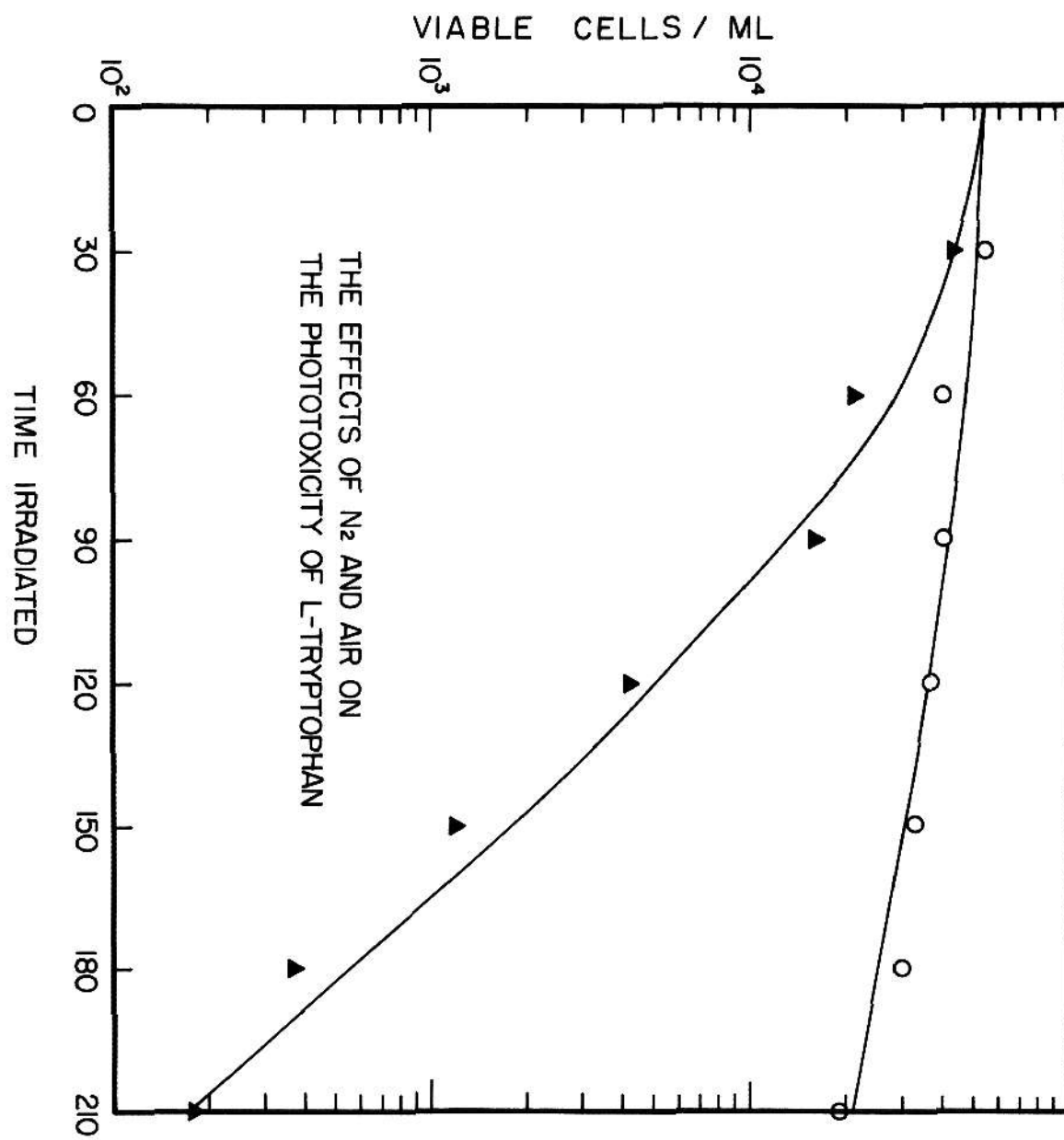


Figure 3



### Explanation






Figure 4 is an overlay of the graphs in Figures 4a thru 4e. The open square  corresponds with Figure 4a, the open circle  corresponds with Figure 4b, the open triangle  corresponds with Figure 4c, the closed square  corresponds with Figure 4d, and the closed circle  corresponds with Figure 4e. Figures 4a thru 4e are plots of the number of KSU 3916 cells surviving a given incubation period in a tryptophan solution which received a prescribed dose of blacklight radiation.



Figure 4

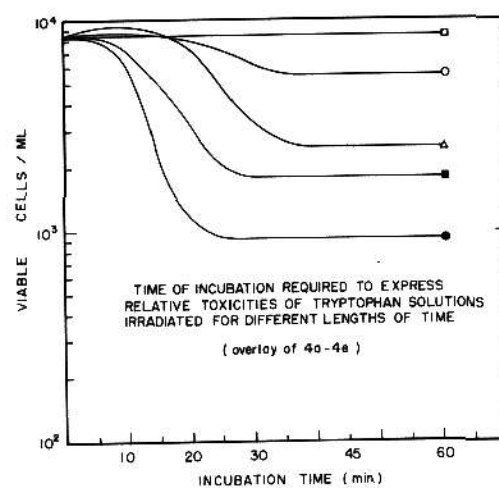


Figure 4a

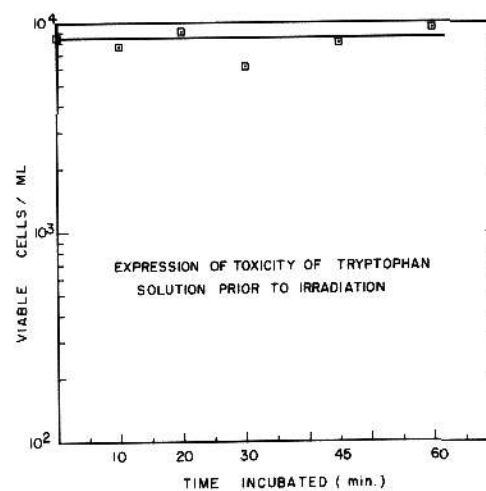


Figure 4b

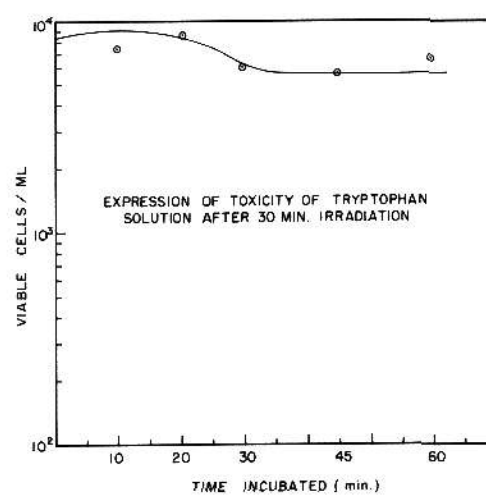


Figure 4c

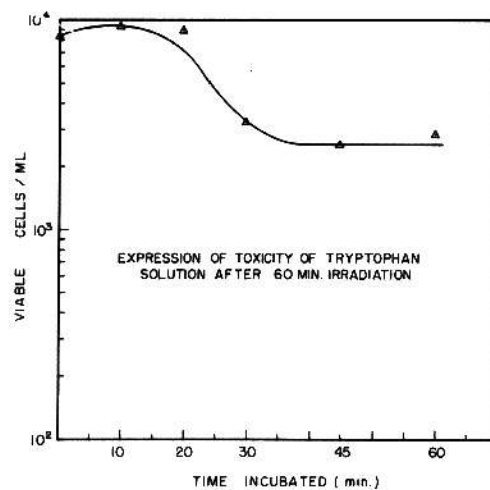


Figure 4d

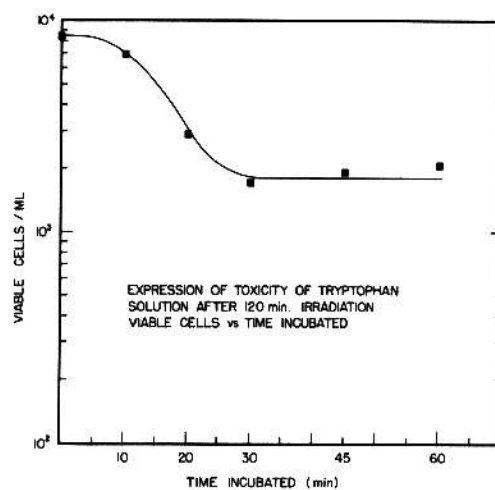
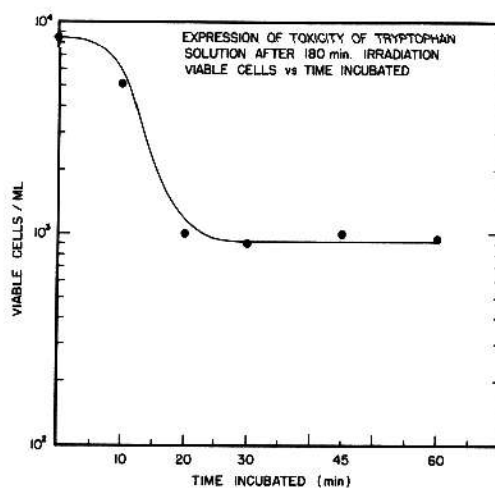


Figure 4e



of % T vs Correction Factor (A.I.D.). Since it was necessary to use several concentrations during the experiment to allow comparison of wavelengths within the absorption spectrum of L-tryptophan with wavelengths outside this region, a concentration factor was calculated by dividing the concentration used at a given wavelength by the lowest concentration used at any wavelength. The "Action" of a given wavelength was calculated as the reciprocal of the Average Incident LD<sub>50</sub> Dose times the concentration factor.

Formula:

$$\text{Action (mm}^2 \text{ erg}^{-1}) = 1 \div \text{ave. inc. LD}_{50} \text{ Dose} \times \text{Conc. Factor}$$

The results in Figure 6 shows a definite peak of action at 290 nm with a curve which is skewed into the near-UV region. Results in Figures 6a, 6b, and 6l indicate that the Action Spectrum limits are 260 nm and 370 nm, as these wavelengths showed no capability to cause production of the phototoxin measured. It is interesting to note that while the Action Spectrum curve is definitely skewed toward longer wavelengths far out of the tryptophan absorption spectrum, the action at these wavelengths is 100 to 1000 fold less than the action at 290 nm. The observation that wavelengths outside the absorption spectrum of the irradiated substance are more effective than wavelengths within the absorption spectrum is possibly a violation of the assumption that quantum yield is equal for all wavelengths. It could also imply that the reaction requires more than one wavelength, in which case the longer wavelengths are producing secondary effects with very small amounts of light from the peak. This explanation would fit well with the observation that UV wavelengths shorter than 270 nm are incapable of producing toxin, since the amount of scattered light of wavelengths longer than 310 nm would be practically zero

for these monochromator settings. The observation that 290 nm is more active than 280 nm could be a result of a dual wavelength, or it could suggest that the photoreaction is the result of group specific absorption rather than molecular absorption. This would explain why 290 nm, which is the secondary peak in the absorption spectrum, is the only peak in the Action Spectrum.

The peak of action at 290 nm, and the skewed shape of the curve with an extension well into the near-UV region (310 nm - 360 nm), suggests that the phototoxicity of irradiated tryptophan may be at least partially responsible for the near-UV sensitivity of recA cells. A clear separation of medium and intracellular light toxicity of irradiated tryptophan will be a difficult question to answer, and will probably not be understood until the mechanism of toxicity, and in vivo isolation of phototoxin have been accomplished.

Table 6 shows that the phototoxic reaction is independent of the dose rate, and that the concentration is directly related to the reaction rate. This means that if one half the concentration is exposed to twice as great a dose as twice the concentration, the samples have the same amount of toxic product. This indicates that the concentration may be varied within the range used and compensated for by a concentration factor calculated as described.

#### ix) Mutagenicity Test

The results in Table 7 show that the number of mutants in the continuous culture population increases rapidly after the addition of irradiated tryptophan. While a four to five fold increase in two hours is the largest increase in  $T_5$  resistant cells which could be expected if the observed increase were due to a preexisting background of  $T_5$  resistant cells in the population, an eighteen fold increase is observed for this period, eliminating the possibility that the increase in  $T_5$  resistant cells is due to a preexisting resistant fraction of

the population. It is also shown in Table 7 that exposure of the population to irradiated tryptophan between hour 2 and 4.5 resulted in a fifteen fold increase in  $T_5$  resistant cells, and a contamination at time zero with the addition of the irradiated tryptophan could only account for a six fold increase during this time period. Therefore the data in Table 7 indicate that the irradiated tryptophan solution contains a mutagen. Some indication as to the strength of this mutagen is obtained by the observation that after twenty-six hours of exposure of the population to the mutagen, the number of  $T_5$  resistant mutants levels off at approximately 10 percent of the population.

The ability of the irradiated tryptophan solution to cause mutations at a reasonably high rate, 1.0 percent of the population for a given marker, suggests the possibility that its primary site of action in the cell could be the DNA. If this were the case the mechanism of mutagenesis would provide an important insight into the rec system, as the rec mutants are much more sensitive to this toxin than other near-UV sensitive mutants. However, the possibility that the toxic product is not the same substance which is mutagenic has not been eliminated.

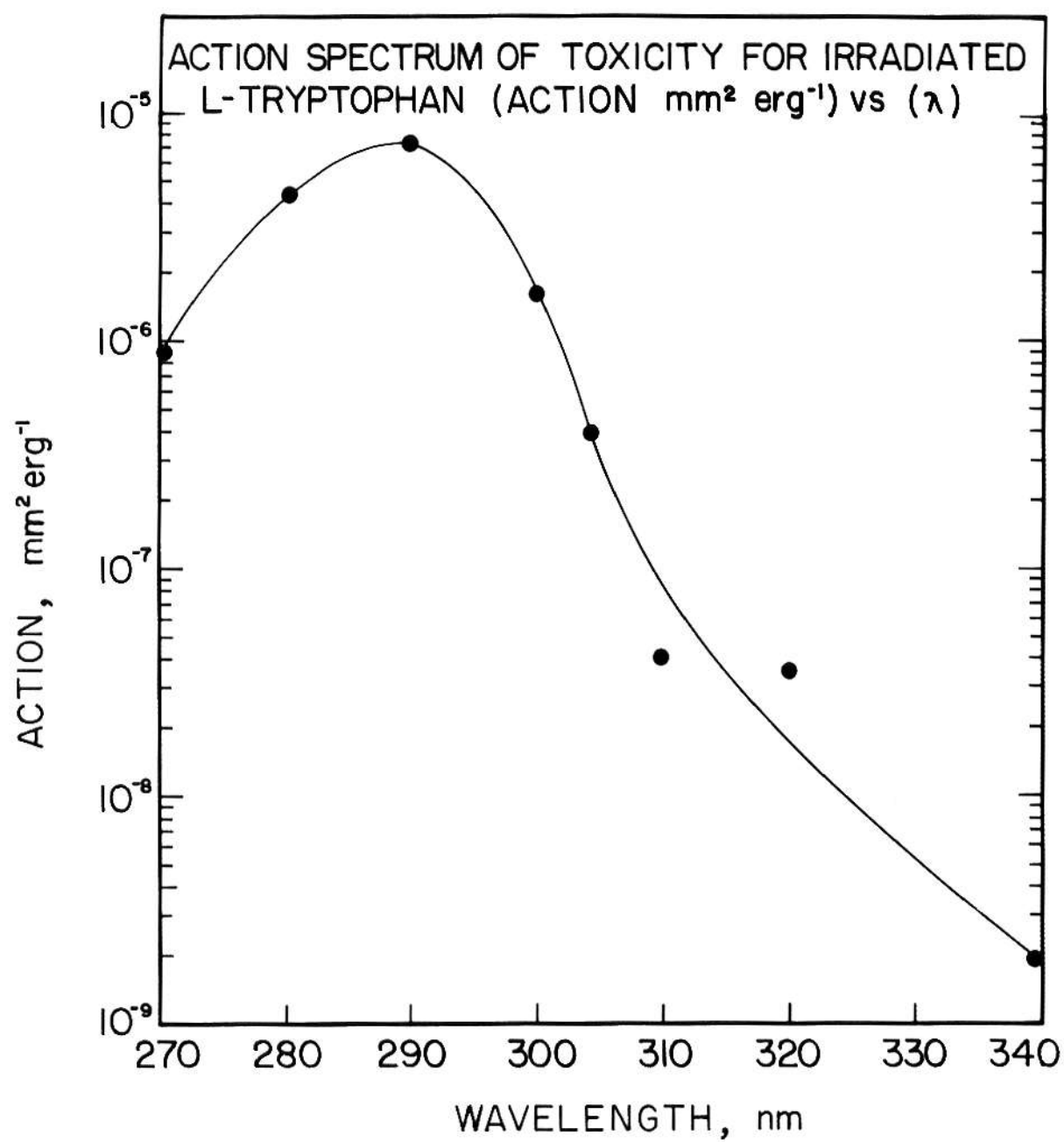


### Explanation

Figure 5 is an action spectrum of the toxicity of irradiated L-tryptophan plotted as Action ( $\text{mm}^2 \text{ erg}^{-1}$ ) vs wavelength. The Action of a particular wavelength is a quasi-rate constant of the production of toxic photoproduct required to kill 50% of a test population of KSU 3916 cells. Therefore, the points plotted in Figure 5 were determined using the corrected  $\text{LD}_{50}$  dose extrapolated from the graphs in Figures 6a thru 6l. The formula used to calculate the Action of a given wavelength is:

$$\text{Action } (\text{mm}^2 \text{ erg}^{-1}) = 1 \div \text{ave. inc. } \text{LD}_{50} \text{ dose} \times \text{conc. factor.}$$

Figure 5







### Explanation

Figures 6a thru 6l are plots of the number of KSU 3916 cells which survived 30 minutes incubation with a solution of L-tryptophan (concentration listed) irradiated with a particular wavelength of light versus the length of time this solution of L-tryptophan was irradiated. The dose of a particular wavelength required to kill 50% of the test population was extrapolated from these graphs and used to calculate the Action of each wavelength for the Action Spectrum plotted in Figure 5.

Figure 6a

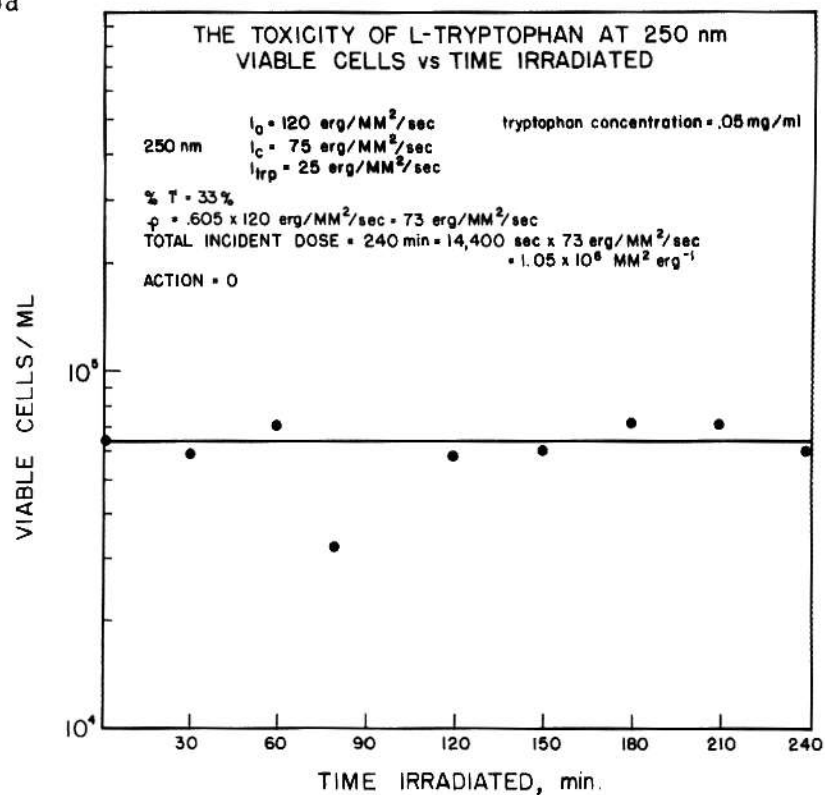


Figure 6b

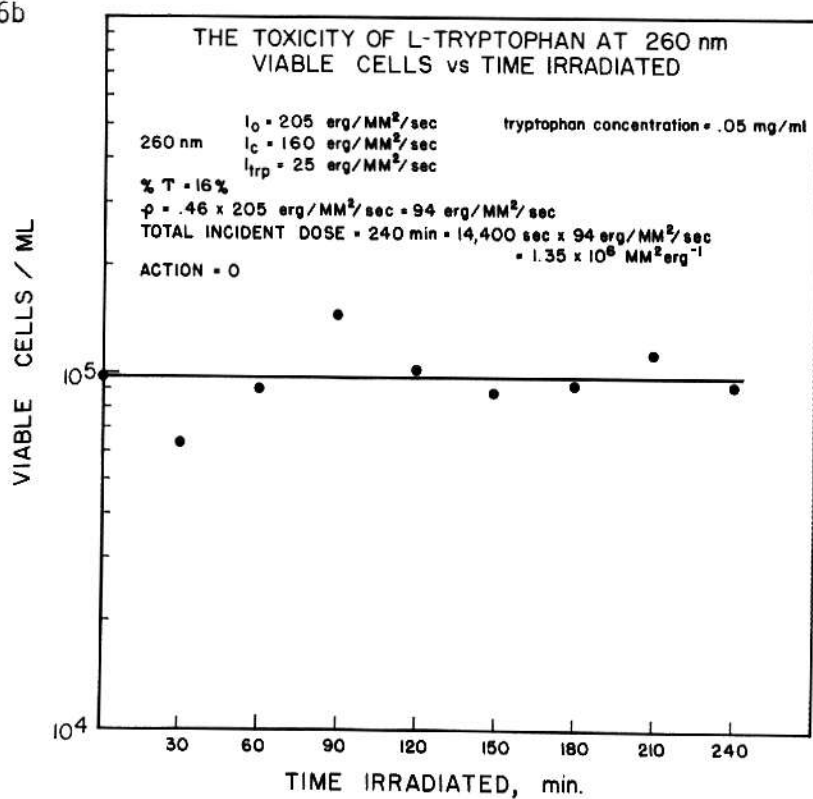


Figure 6c

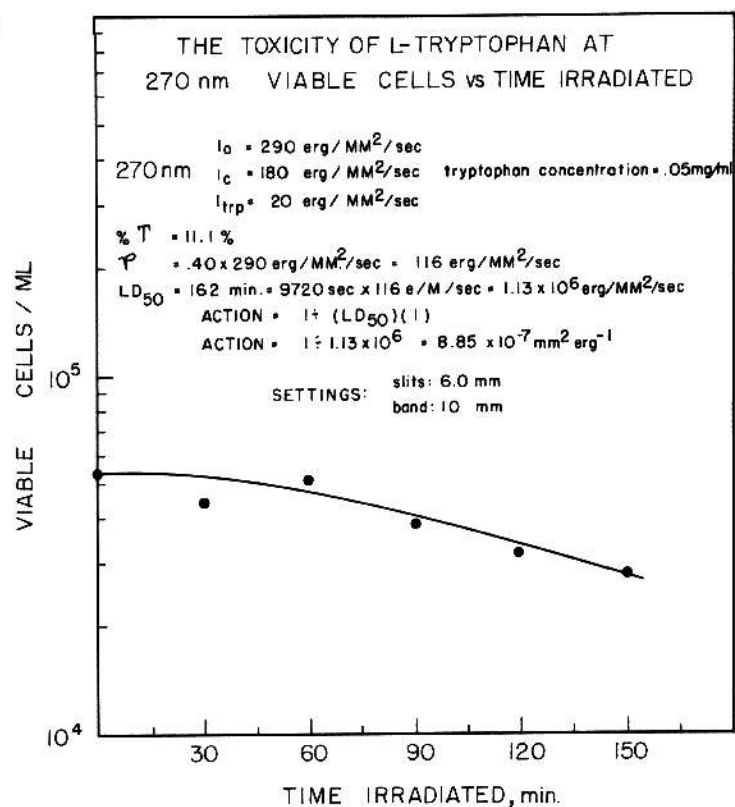


Figure 6d

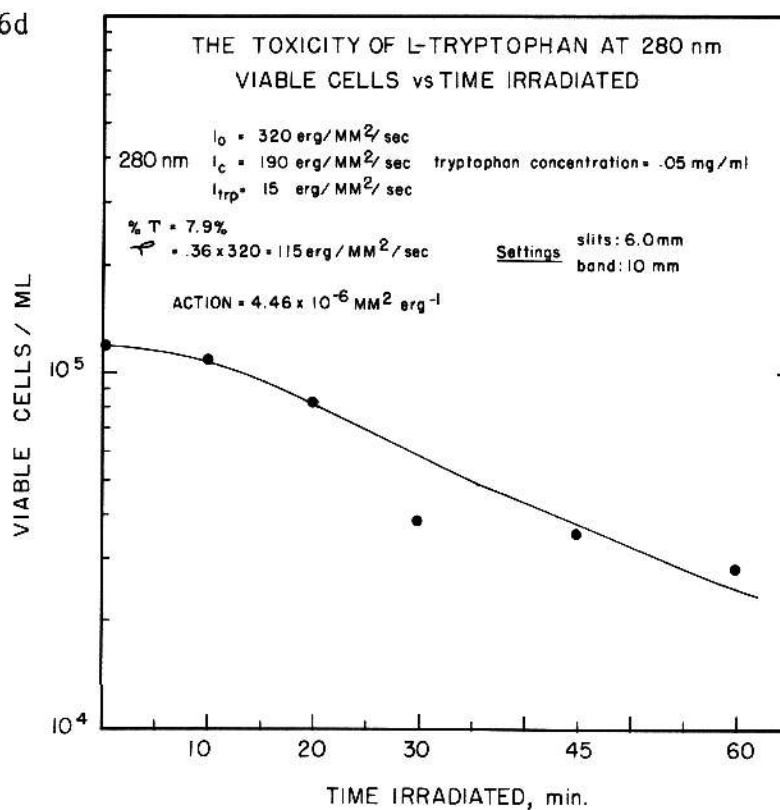


Figure 6e

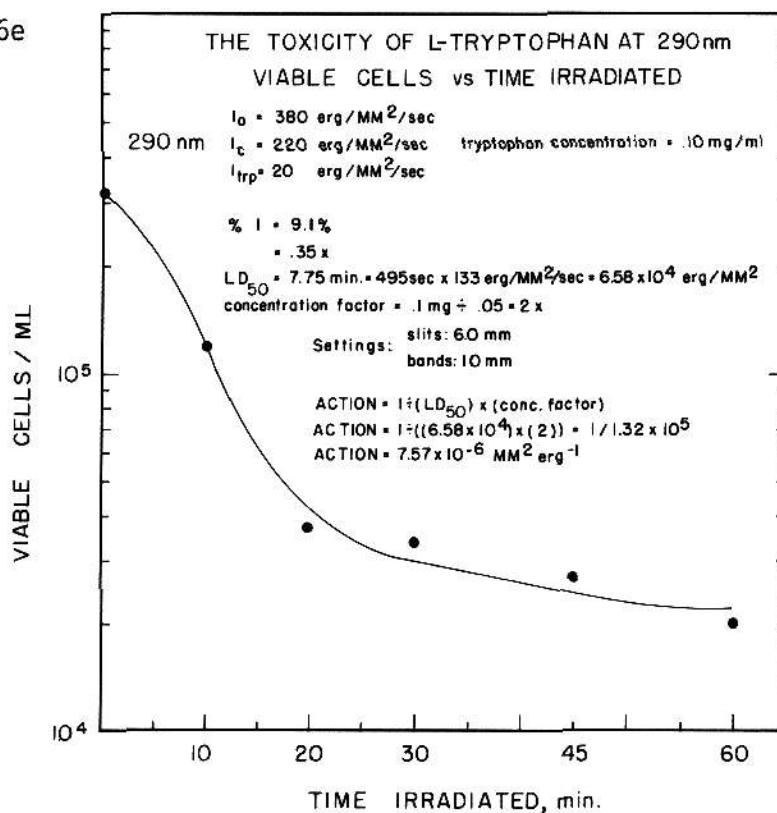


Figure 6f

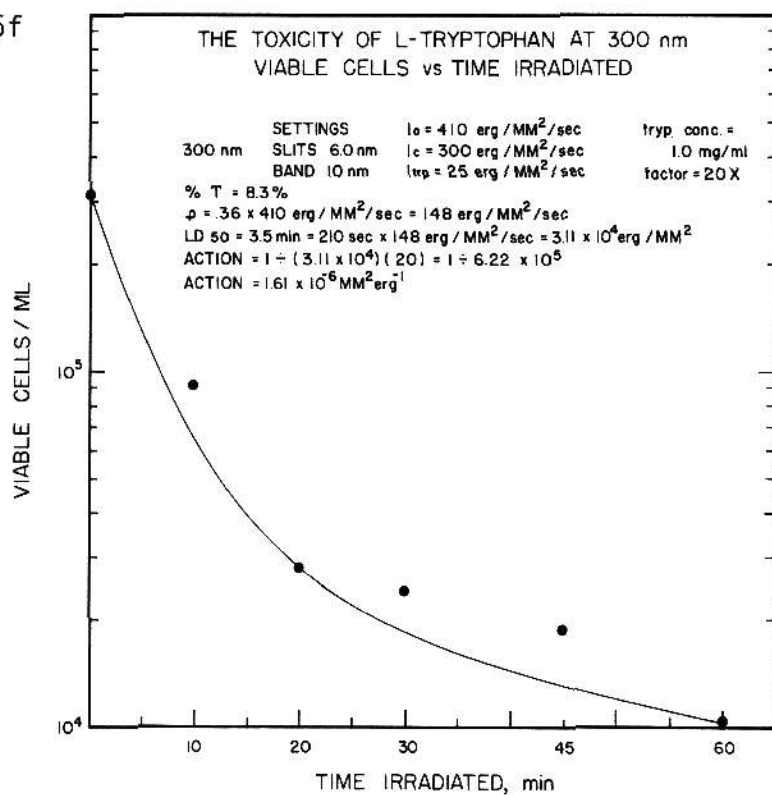


Figure 6g

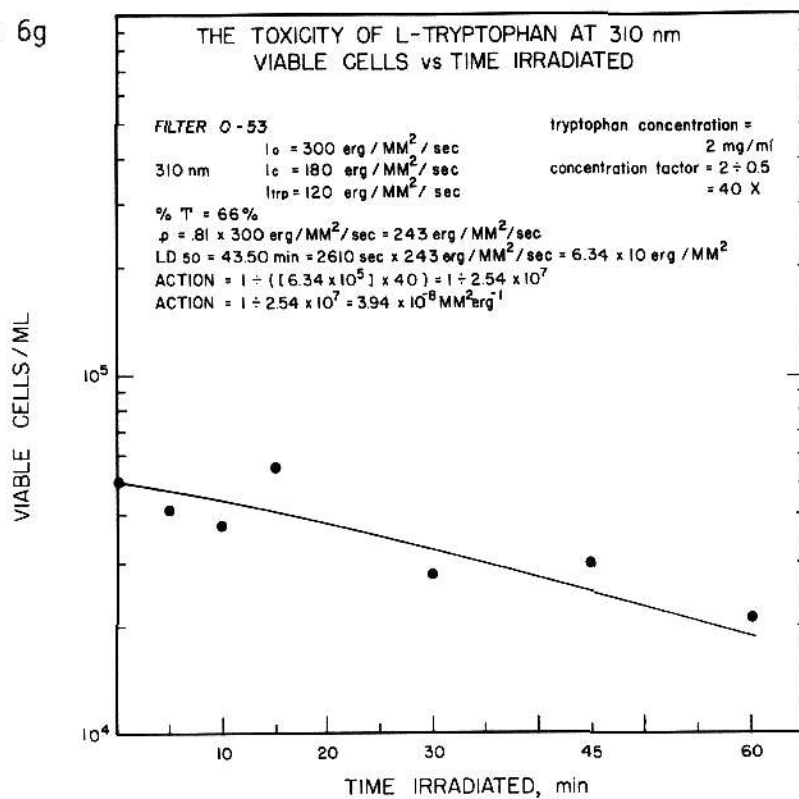


Figure 6h

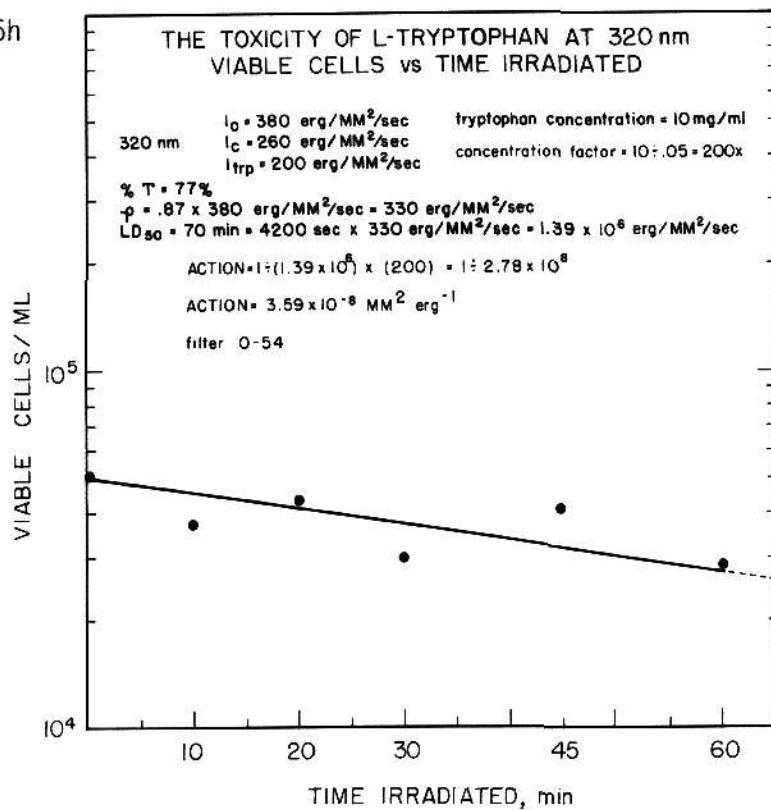


Figure 6i

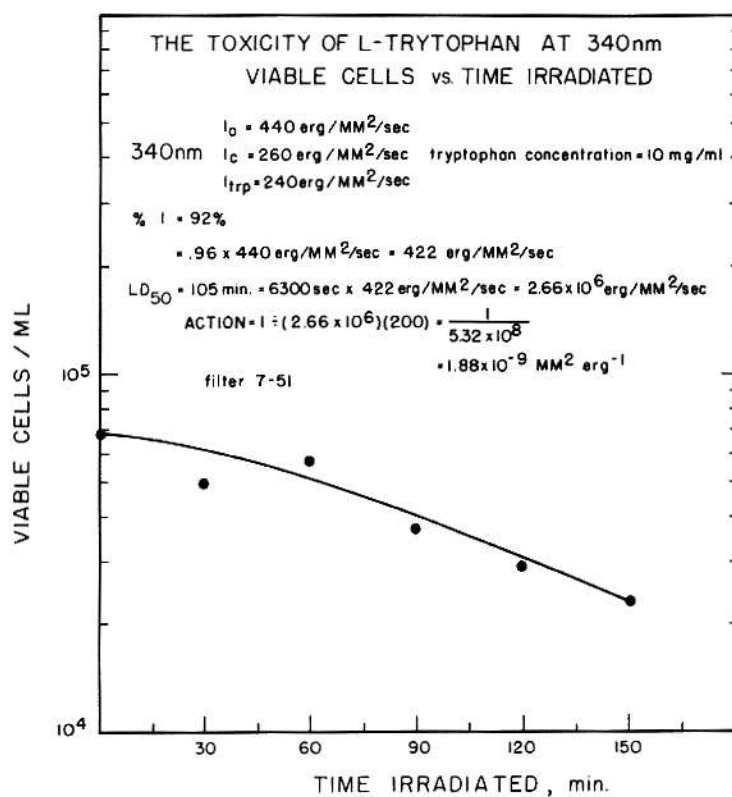


Figure 6j

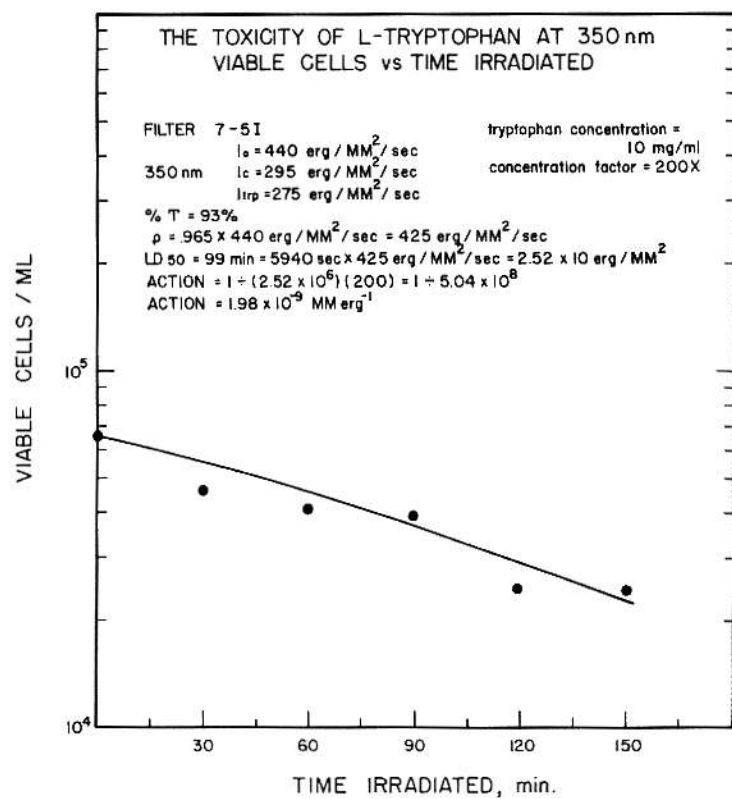


Figure 6k

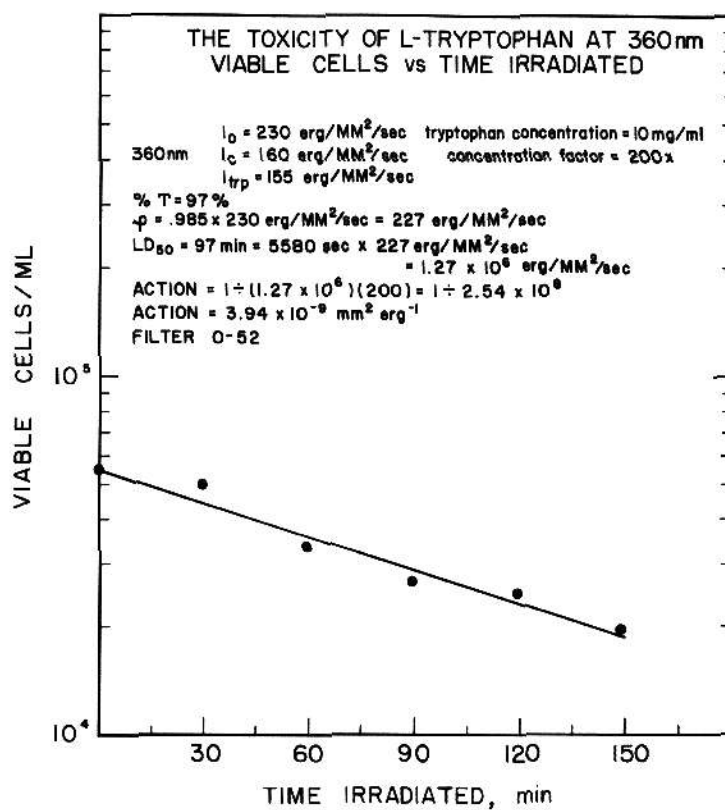


Figure 6l

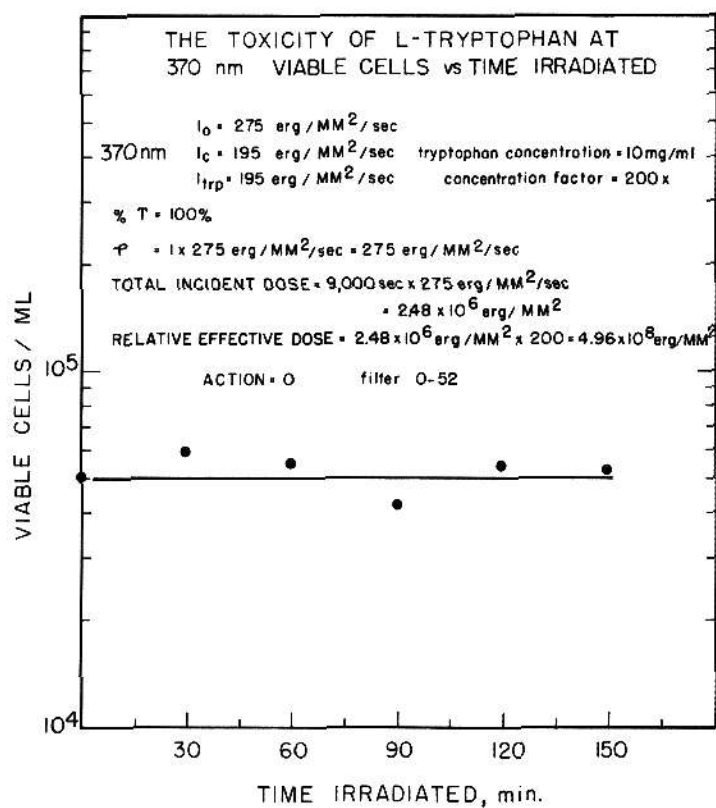




Table 6. Action Spectrum Reciprocity of Dose and Concentration at 280 nm and 320 nm (Survivors  $\times 10^4$ )

Multiplicity of Dose 280 nm	Concentration of Tryptophan (mg/ml)			Exposure Time (min)
	.05 (1/1)	.025 (1/2)	.01 (1/5)	
0	9.6	9.3	9.1	0
1/1	<u>5.3</u>	7.6	8.4	30
2/1	1.8	<u>5.4</u>	8.0	60
5/1	.95	5.3	* <u>8.1</u>	150
320 nm	10 (1/1)	5 (1/2)	2 (1/5)	
0	7.8	7.5	8.1	0
1/1	<u>5.9</u>	7.0	7.8	30
2/1	3.5	<u>5.7</u>	7.0	60
5/1	1.2	4.3	<u>5.5</u>	150

#### Explanation

The values listed here are the averages of four plate counts for each listing. Comparison of the underlined listings indicates that with the exception of the 1/5\* dilution at 280 nm lower concentrations of substrate are compensated by larger doses of light. This Table also indicates that within the range of concentrations used for this experiment a concentration factor may be calculated, and that the effect observed is a result of the total dose and not the dose rate.

Table 7. The Appearance of T5 Resistant Mutants in a Continuous Culture of E. coli B/r/t<sup>-</sup> upon Exposure to Irradiated L-tryptophan

Time (hrs)	Viable Count (x10 <sup>7</sup> )	T5 Resistant Mutants	T5 Resistant Mutants/10 <sup>7</sup> Cells
0	4.4	1.5 x 10 <sup>2</sup>	46
2	2.6	2.8 x 10 <sup>3</sup>	864
4.5	4.0	4.6 x 10 <sup>3</sup>	1.42 x 10 <sup>3</sup>
6.5	2.0	6.1 x 10 <sup>3</sup>	1.88 x 10 <sup>3</sup>
7	1.8	9.9 x 10 <sup>3</sup>	3.05 x 10 <sup>3</sup>
8	2.4	2.15 x 10 <sup>4</sup>	6.66 x 10 <sup>3</sup>
9	2.8	1.62 x 10 <sup>4</sup>	5.00 x 10 <sup>3</sup>
18	3.8	2.12 x 10 <sup>4</sup>	6.54 x 10 <sup>3</sup>
19	2.6	2.05 x 10 <sup>4</sup>	6.33 x 10 <sup>3</sup>
20	3.2	1.70 x 10 <sup>4</sup>	5.25 x 10 <sup>3</sup>
22	3.4	2.12 x 10 <sup>4</sup>	6.54 x 10 <sup>3</sup>
24	2.6	2.75 x 10 <sup>4</sup>	8.49 x 10 <sup>3</sup>
26.5	3.4	3.13 x 10 <sup>4</sup>	9.66 x 10 <sup>3</sup>
44	4.2	3.98 x 10 <sup>4</sup>	1.23 x 10 <sup>4</sup>
46	4.0	3.88 x 10 <sup>4</sup>	1.20 x 10 <sup>4</sup>
49	4.6	3.92 x 10 <sup>4</sup>	1.21 x 10 <sup>4</sup>
Ave. = 3.24 x 10 <sup>7</sup>			

#### Explanation

The listings for "viable counts" and "T5 resistant mutants" are the averages of four platings. The number of T5 resistant mutants per 10<sup>7</sup> cells was determined using the average of all viable counts and the number of T5 resistant cells at each time.

## SUMMARY

These experiments suggest that at least part of the lethal effect of near-UV radiation for recA mutants is due to the production of a phototoxin by the photooxidation of L-tryptophan in the medium and within the cell. The toxicity observed could be directly related to the recA lesion; as E. coli B phr<sup>-</sup> mutants, which are more sensitive to visible light than recA mutants, are not killed by the tryptophan phototoxin and are not as sensitive to near-UV irradiated nutrient agar plates as recA mutants (R. B. Webb, personal communication). This would suggest the possibility that near-UV light produces more than one phototoxin, but that wildtype cells are capable of repairing this damage. Therefore, this phototoxin is probably only one of several near-UV phototoxins which are specifically effective for a particular near-UV sensitive mutant.

The mutagenicity of this phototoxin indicates that there is an indirect mechanism by which near-UV light can damage the DNA of the cell, and raises the possibility that the recA lesion includes the inability to repair this particular damage. The mechanism of mutagenicity of wavelengths longer than 290 nm will have important implications for such medical problems as xeroderma pigmentosum, a human disease in which the skin is very sensitive to sunlight because of its inability to repair DNA (9) (10). The possibility that this sensitivity could be due to near-UV phototoxic effects should be considered, since a reasonable amount of light in the near-UV region reaches the earth while almost all 260 nm UV is filtered out by a layer of ozone in the upper atmosphere (2).

This group of experiments suggests that near-UV effects can be screened for and characterized individually, and indicates that a set of experiments

to identify and characterize several of these phototoxins would provide important information about near-UV light effects.

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PHOTOTOXICITY OF NEAR-UV IRRADIATED L-TRYPTOPHAN  
FOR RECOMBINATIONLESS MUTANTS OF SALMONELLA TYPHIMURIUM

by

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## ABSTRACT

After radiation of several substances, it was found that near-UV irradiation of L-tryptophan causes the production of a phototoxin which was preferentially toxic for recA mutants. This toxic solution was then tested for stability to acid and basic hydrolysis, and analyzed on a Beckman 120C Amino Acid Analyzer for the appearance of new amino acid peaks. A number of organic solvents were tested for the ability to extract the phototoxin from the exposure solution, and a cyclohexanone extract was scanned for fluorescence after extraction. The phototoxic reaction was then tested for oxygen requirement by radiating two samples, one of which was purged with  $N_2$  and the other purged with air and it was found that  $N_2$  prevented the reaction. The time required for expression of relative toxicity to a test cell population was determined by irradiating a tryptophan solution with different doses and then sampling a population of cells incubated in these solutions at several time intervals. An action spectrum of toxicity was done to determine what wavelengths of light would produce the phototoxin, and what wavelength was most effective for production of the phototoxin. Finally, the phototoxic solution was tested for the ability to cause E. coli B/r/t to mutate to  $T_5$  resistance, and found to express mutagenic properties.