ACTION SPECTRA OF LETHALITY IN A RECOMBINATIONLESS MUTANT OF SALMONELLA TYPHIMURIUM

by

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B. S., Kansas State University, 1970

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

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THIS BOOK CONTAINS NUMEROUS PAGES WITH THE ORIGINAL PRINTING BEING SKEWED DIFFERENTLY FROM THE TOP OF THE PAGE TO THE BOTTOM.

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INTRODUCTION

Bacterial mutants of Salmonella typhimurium that are unable to undergo genetic recombination have been found to be highly sensitive to light emitted from blacklight and fluorescent bulbs (Eisenstark and Ruff, 1970). These recA mutants are also sensitive to ultraviolet (UV), X-rays, and chemical mutagens (Eisenstark, et al., 1969). Further screening of other bacterial strains has shown that this increased sensitivity to near-UV light is a unique characteristic of recombinationless mutants. Lethality by monochromatic light above 300 nanometers (nm) has definitely been established, thus eliminating the possibility that the killing is the result of stray far-UV that is emitted from the blacklight and other fluorescent bulbs (Webb and Lorenz, 1970).

It is known that pyrimidine dimers are produced in the DNA of bacteria as the result of the absorption of far-UV (254 nm). Although pyrimidine dimers are not the only photoproducts formed in the DNA by UV light they probably cause most of the lethality (Smith, 1966). It is unknown, however, what the chromophore(s) is that absorbs the near-UV radiation or that passes the energy on to other molecules and thus causes lethality. Since the recA mutants are extremely sensitive, the light chromophore(s) may have a role in recombination and repair.

An action spectrum is the relative biological or chemical response to different wavelengths of light. It has been found that a plot of the effectiveness of various wavelengths of UV in killing bacteria shows a maximum around 260 nm. This action spectrum corresponds in the shape of the curve and in its peak of sensitivity to the absorption spectrum of nucleic acids. The chromophore absorbing the radiation, and thus promoting lethality to the cell, is the

DNA. There are several natural sensitizers in bacteria that may account for the near-UV lethality. The candidates include carotenoids, porphyrins, cytochromes, cytochrome oxidase, nicotinamideadenine dinucleotide (NAD), reduced form of NAD (NADH), flavins, heme proteins, and other pigmented components (Eisenstark, 1970).

The purpose of this investigation was to determine if an action spectrum of lethality would correspond to the absorption spectrum of one of the above compounds. An action spectrum of the lethality caused by monochromatic light at selected wavelengths between 240 and 550 nm was determined with a recombinationless strain of S. typhimurium. It was realized at the initiation of these studies that the chromophore responsible for the near-UV effect might be present in very trace amounts and subsequently be overlooked as a candidate. Two other problems that might hinder this investigation are the possibilities that: 1) more than one chromophore is responsible for the response; or 2) more than one wavelength must be absorbed by the chromophore to affect a response.

A comparison of the survival curves for both stationary and logarithmically growing cells were made to determine if there is any difference. A direct relationship between the degree of sensitivity and the state of cell division was observed (log phase more sensitive than stationary phase) when the source of energy were blacklight bulbs with spectra that peak at 365 nm (Eisenstark, 1970).

REVIEW OF LITERATURE

Definition of Action Spectrum

Action spectroscopy, which is the measure of the dependence of a phenomenon to various wavelengths, is an important tool that is used in photobiology to help identify the active absorbing molecule. This technique is not new but has been used for almost a century in the identification of chromophores for photosynthesis (French and Young, 1956). That few action spectra appear in the literature reflects the fact that reliable action spectra are not easy to obtain because of the variables in living systems which affect the efficiency of a radiation induced response. Also a particular action spectrum is very specific for the conditions at which the response of a biological system to the radiation is observed. Varying the conditions may vary the action spectrum of the phenomenon.

Absorption spectra are not fixed. The absorption by the various chromophores depends upon the composition of the solvent and the structure of the molecule. The absorption spectra are sensitive to changes in pH especially in the regions of the pK values of the functional groups attached to the purines and pyrimidines. In the far-UV effective absorbers are six-membered carbon rings (benzene, toluene, phenol), rings containing nitrogen (pyridine, pyrimidine, imidazole), various double rings (naphthalene, purine, indole), triple rings (anthracene, flavin), and quadruple rings (steroid, porphyrin) all of which have conjugated double bonds. In the near-UV region the effective absorbers are three ring molecules such as riboflavin or four rings such as some steroids, all porphyrins, and long-chain conjugated molecules such as carotenoids (Jagger, 1967).

The basic assumptions underlying the use of action spectra in biology that were established by Loofbourow in 1948 are still pertinent today. They are as follows: (1) the observed photobiological response is the consequence of a photochemical reaction of molecules; (2) the quantum efficiency is independent of wavelength over the spectra span studied; (3) scattering, reflection, and absorption of the radiation within a cell by inactive substances before it reaches the chromophore is either negligible or constant at all wavelengths; (4) at no wavelength of interest is all of the incident radiation absorbed by the sample; (5) the reciprocity law holds for the range of exposure times used in the experiments.

This review of the literature on action spectra will not cover the research that has been done on photosynthesis, phototropism, and other processes exhibited by plants. Excellent reviews of these areas can be found in Radiation Biology volume III (Holleander, 1956) and in Photophysiology volume 1 (Giese, 1964).

Action Spectra Identifying Nucleic Acids as the Genetic Material

At the beginning of this century there was great speculation as to the molecular nature of the carrier of hereditary information. An action spectrum provided some of the first evidence that nucleic acids are the vital genetic material of all living organisms. Gates in 1930 published action spectra for the killing of Escherichia coli and Staphylococcus aureus by monochromatic far-UV radiation at wavelengths from 230 to 295 nm. Comparison of different survival curves were made by plotting the inverse of the incident dose required to produce a constant amount of inactivation. Gates demonstrated that the bactericidal action spectra closely matched the absorption spectra of deoxyribonucleic acid with a maximum of 260 nm, a minimum at about 230 nm

with indication of another maximum at wavelengths shorter than 230 nm. Similar action spectra for lethality of bacteria were published by Ehrismann and Noethling (1932) for Pseudomonas pyocyanus, Micrococcus condicans, S. aureus; by Duggar and Hollaender (1934a, 1934b) and Wyckoff (1932) for Serratia marcescens.

The use of action spectra was soon extended to investigate the loss of infectivity or inactivation of virus particles. The early action spectra on inactivation caused by far-UV monochromatic light include vaccinia virus (Rivers and Gates, 1928), Rous sarcoma virus (Sturm et al., 1932), Staphylococcus bacteriophage (Gates, 1934), tobacco mosaic virus (Hollaender and Duggar, 1936), influenza virus (Hollaender and Oliphant, 1944), T_1 bacteriophage (Fluke and Pollard, 1949), Bacillus megatherium bacteriophage (Franklin et al., 1953), and T_1 and T_2 bacteriophage (Zelle and Hollaender, 1954). These can now be divided into two groups. Tobacco mosaic virus, Rous sarcoma, and influenza virus contain RNA and the second group which includes the rest of the above contain DNA. Absorption spectra of native DNA and RNA are indistinguishable except for molecules with unusual base ratios because three of the four bases are the same (Jagger, 1967).

Early action spectra of fungi and plants that indicate the importance of DNA should also be mentioned. Action spectra of the delay in the budding phenomenon and the killing of the yeast Saccharomyces cerevisiae (Wyckoff and Luyet, 1931; Ehrismann and Noethling, 1932; Oster, 1934a, 1934b, 1934c) follow the absorption spectrum of DNA as does the killing of spores of Trichophyon mentagrophytes (Hollaender and Emmons, 1939). The spores of Rhizopus suinus show a similar behavior of delay germination and killing as well as somewhat retarded growth rate of the germinated spores (Dimond and Duggar, 1940). Inactivation spectra for pollen grains of maize (Stadler and Uber, 1942), spores of

Ustilago zeae (Landen, 1939), and spores of Aspergillus niger (Zahl et al., 1939) have a peak at 265 nm.

The chromophore in all of the inactivation studies absorbing the energy are the heterocyclic bases, the purines and pyrimidines forming the polymer DNA. The individual bases have different absorption spectrum which do not correspond to the action spectrum of DNA (Sinsheimer, 1954). It was not until 1960, however, that Beukers and Berends discovered that pyrimidine dimers are the photoproducts in the DNA produced by UV irradiation which account for most of the lethality.

Many action spectra on the responses of organisms to far-UV other than lethality have been published in which nucleic acids are the absorbing chromophore. Some of the more interesting ones are retardation of the rate of regeneration of Blepharisma (Giese et al., 1956; Hirshfield and Giese, 1953), retardation of division of sea urchin eggs fertilized with irradiated sperm (Giese, 1939), inhibition of hatching of Habrobracon eggs after irradiation of the nuclear side (Goldman and Setlow, 1956), inhibition of growth of tissue cells in culture (Mayer and Schreiber, 1934), chloroplast loss in Euglena (Lyman et al., 1959), phage induction in E. coli K-12 (λ) (Franklin, 1954), and numerous ones on mutation induction which will be discussed separately below.

Action Spectra in which Protein is the Chromophore

The absorbing molecules in proteins are principally the aromatic amino acids resulting in an absorption maximum around 280 nm. Tryptophan and tyrosine are the major absorbers of far-UV above 250 nm but phenylalanine, cystine, and cysteine are also important. Disulfide bonds are necessary for

maintaining the structural integrity of the protein molecule, so that their presence increases the UV lability of the protein. The peptide bond is a relatively weak absorber of UV but the collection of all peptide bond in a protein molecule makes it an important absorber of UV below 240 nm. While the absorption spectra of nucleic acids are very similar the absorption spectra of proteins are not as clear cut because of variations in content of aromatic amino acids (Jagger, 1967).

A number of biologically observed effects are correlated to an action spectrum with a peak of 280 nm and a minimum at 250 nm indicating proteins as the chromophores. Examples are: immobilization and cytolysis of paramecia (Giese and Leighton, 1935); prevention of hatching of nematode eggs (Hollaender, et al., 1940; Jones and Hollaender, 1944; Jones, et al., 1940); retardation of excystment of the ciliate Colpoda (Giese, 1941); sensitization of Paramecium to heat (Giese and Crossman, 1945); retardation of division of sea urchin eggs fertilized with normal sperm (Giese, 1939); and inactivation of the hemagglutination capacity of influenza virus (Tamm and Fluke, 1950). It now seems probable that in some of these cases DNA-protein linkages are involved.

Recently the destruction of the mitotic spindle has been attributed to a protein in the cytoplasmic pool. Irradiation of the cytoplasma adjacent to the mitotic spindle causes the spindle to diminish or disappear. Action spectrum of this spindle destruction in salamander tissue culture cells irradiated in early metaphase and the action spectrum for anaphase delay parallel the absorption spectrum of a tyrosine containing protein (Brown and Zinkle, 1967).

It is interesting to note cases in which different functions in the same organism have different action spectra. For influenza virus the action spectrum

for its infectivity is similar to the absorption by nucleic acids, whereas the action spectrum for inactivation of its hemagglutination capacity is comparable to the absorption spectrum for a protein (Tamm and Fluke, 1950). The action spectrum for the immobilization of ciliary movement and sensitization to heat in Paramecium resembles the absorption spectrum for a protein (Giese and Crossman, 1945; Giese, 1945a) while the action spectrum for the retardation of division of the same cells resembles nucleic acid absorption (Brandt and Giese, 1956). Similarly, the action spectrum for retardation of cell division in the eggs of the purple sea urchin resembles protein absorption when the eggs are irradiated and nucleic acid absorption when the sperm are irradiated. It is thought that the effect on the egg might be accounted for by damage to the cytoplasm which shields the nucleus whereas the nucleic acid is directly affected when the sperm are irradiated (Giese, 1939).

When interpreting a far-UV action spectra the question may arise of whether the chromophores absorbing the energy are nucleic acids, proteins, or both. A comparison of the ratio of effectiveness at 260 nm to that at 280 nm should be determined. DNA in 0.10 M phosphate buffer at pH 7 has a 260/280 nm ratio of optical density of about 1.85. Action spectra showing lower ratios than this probably involve some protein and certainly do if the ratio is less than one. Also a comparison should be made of the minimum of the action spectrum. Nucleic acids have a characteristic minimum at 230 nm, while proteins have a minimum around 240 or 250 nm. At 230 nm protein absorption is usually high (Jagger, 1967).

A very accurate method of determining nucleic acid or protein action has been suggested by Epstein and Schiff (1961). They use a polychromatic light source to determine the relative efficiencies for the killing of T_2 phage with and without a filter that cuts off around 280 nm. The inactivation of T_2 by

the two sources (filtered and unfiltered) yield curves differing only in the time scale. The curves coincide when the time of exposure to the unfiltered light is multiplied by 10. Ten seconds of filtered UV is required to produce the same amount of killing with one second of unfiltered UV. The killing which is a result of DNA absorption gives a dose rate of 10:1. The procedure is then repeated for DNAse, a typical protein which gives a dose ratio of 3.5:1. These two ratios serve as the standards for comparison. An unknown system is inactivated by exposure to both the filtered and unfiltered light sources and the dose ratio is determined. This ratio is compared with the two standard ratios to determine if the chromophore is protein or nucleic acid. This method is quite sensitive, since the peak absorptions of proteins and nucleic acids are separated by only 20 nm but they yield widely different dose ratios.

Action Spectra in which Protein Linked to DNA is the Chromophore

Action spectra may indicate that protein cross-linked to DNA is the absorbing unit in the reaction. Action spectrum for thymine dimerization in Micrococcus radiodurans, an organism that is extremely resistant to irradiation, shows that the formation of dimers has a maximum at 260 nm and that the frequency of formation is the same as in most bacteria strains. The action spectra for killing and delay in DNA synthesis are similar to one another but do not correspond to the absorption spectrum of DNA. They show a high component of sensitivity at 280 nm as well as 260 nm. It is suggested that the resistance of this organism to UV is due to its ability to repair thymine dimers, but what ultimately kills the organism is damage to the protein crossing-linking with the DNA (Setlow and Boling, 1965).

Work with mammalian cells also indicates the importance of proteins as chromophores. When a segment of the chromosomes is exposed to irradiation a

phenomenon occurs known as paling. Under a medium dark phase microscope these chromosomes appear gray or even clear whereas nonirradiated chromosomes appear black. The change in appearance is due to a loss of nonaqueous material. The conclusion from the results of action spectrum studies is that paling is most likely due to loss of both protein and DNA in combination (Zirkle and Uretz, 1963).

The action spectrum for inactivation of a Rhizobium bacteriophage by ultraviolet wavelengths follows the absorption spectrum of DNA at wavelengths between 240 and 290 nm. Below 240 nm the action spectrum deviates sharply upward and corresponds to protein absorption. Inactivation may depend on damage to both the nucleic acid and the protein of the phage (Kleczkowski and Kleczkowski, 1965).

Action Spectra on Mutation Induction

Original mutation studies using nonionizing radiation were done on Drosophila and pollen grains. Difficulties became apparent, however, when an attempt was made to determine the relative mutagenic effectiveness of different wavelengths. Some of the UV radiation was absorbed by the cell wall or layers of cells before it reached the genetic material, therefore the action spectra were not accurate.

In 1939 Knapp and co-workers obtained an action spectra of mutation induction in irradiated spermatozooids of the liverwort Sphaerocarpus donnellii whose thin cell wall absorbs little UV. They found a maximum of mutation induction at 265 nm similar to the maximum absorption of DNA. At the same time Hollaender and Emmons (1939) were investigating induced mutations characterized by a difference in kind or degree of pigmentation, in growth rate, or a difference in the amount of aerial hyphae for the dermatophyte Trichophyton

mentagrophyte isolated from dermatophtosis. Their action spectra for mutation induction also corresponds to the absorption spectra of nucleic acid. Action spectra studies on mutation induction were extended to other fungi. Penicillium notatum (Hollaender and Zimmer, 1945; Hollaender and Schoeffel, 1931), Neurospora crassa (Hollaender and Sansome, 1945), and Aspergillus terreus (Hollaender et al., 1945), all have a peak at 260 nm.

The first action spectrum for UV mutagenesis in bacteria was published by Kaplan in 1952. He looked at three types of mutation using five monochromatic wavelengths between 248 and 303 nm. Again, a maximum of efficiency of mutation induction was observed at 265 nm. This finding was verified with the bacteria E. coli B/r for mutation both from streptomycin dependence to independence and from purine dependence to purine independence (Zelle, et al., 1958). These action spectra for mutagenesis had a great impact on genetics because they were obtained at a time when correlations between nucleic acid changes and variability in inheritance were being studied.

At the same time Hollaender was investigating mutation induction in Trichophyton mentagrophyte he observed chromosome aberrations and obtained an action spectrum for the phenomenon. Action spectra for chromosome aberrations have also been obtained for Drosophila and Chinese hamster cells. The most frequent aberrations observed are the simple chromatid breaks and isochromatid aberrations. The action spectrum of their occurrence peaks clearly at 265 nm (Kirby-Smith and Craig, 1957). However, the action spectrum for chromosome aberrations in Chinese hamster cells suggests strong protein involvement (Chu, 1965). In addition to chromatid breaks and isochromatic aberration, translocations are observed in Chinese hamster cells.

Mutation induction studies have been extended to the near-UV and visible regions. Although doses of 10 to 50 times as great as at 265 nm are required,

wavelengths in the range of 290 to 315 nm are mutagenic in Aspergillus terreus (Hollaender and Emmons, 1946). Kaplan (1952) has found sulfanilamide mutants in S. marcescens induced at wavelengths of 310 to 400 nm. Mutation to resistance to bacteriophage T_5 in continuous cultures of E. coli is induced by visible light (wavelengths longer than 409 nm) and by blacklight (300-400 nm) Webb and Malina, 1967). It is still unknown what the chromophore(s) is that absorbs the energy at these wavelengths and transfers it to the DNA to result in a mutation.

DNA Damage and Repair

Many of the biological effects of UV irradiation can be correlated to the production of certain types of photochemical damage in the DNA. As stated previously the major photoproduct of UV irradiation is thymine dimers in which two thymine molecules are linked to each other between their respective 5 and 6 carbon atoms, thus forming a cyclobutane ring. There is a wavelength dependency for the formation and monomerization of the cyclobutane-type thymine dimers, such that after a sufficient dose of UV a photosteady state between monomer and dimer is reached that is characteristic for the wavelength used. The action spectrum for the formation of thymine dimers shows that at the longer wavelengths around 280 nm the formation of the dimer is favored, while at the shorter wavelengths around 240 nm monomer formation is favored (Deering, 1962). The phenomenon known as short-wavelength reversal has been studied extensively in two purified transforming DNA systems, those of Hemophilus influenzae (Setlow and Setlow, 1963) and Bacillus subtilis (Setlow, 1965). This short-wavelength reversal is due to differences in the absorption spectra for thymine and its dimer and in the quantum yields for the formation and splitting of the dimer (Smith and Hanawalt, 1969).

Five other pyrimidine dimers are also known, cytosine dimers, mixed dimers of thymine and cytosine, and uracil containing dimers in RNA (Smith and Hanawalt, 1969). It must be mentioned again that pyrimidine dimers are not the only photoproducts produced by UV irradiation. There are conditions where other types of photoproducts would be more important.

A repair mechanism cannot be determined unless there is some way to turn it off or at least reduce its effectiveness. The search for recovery mechanisms involves changing the conditions of the environment or the use of UV-sensitive mutants that might be deficient in one or more steps in a repair process. At the present time there are three possible modes for dealing with the damaged DNA. They are excision repair, recombination or post-replication repair, and photoreactivation.

Excision repair is a dark repair mechanism in which the defective regions in one of the two DNA strands is excised and then subsequently replaced with normal nucleotides, utilizing the complementary base pairing information on the intact strand. The mechanism known as the "cut and patch model" involves an extensive enzyme system containing exonucleases, endonucleases, polymerases, and ligases. The excision repair system is capable of recognizing a variety of structural defects in DNA (For reviews see Hanawalt, 1968; Howard-Flanders, 1968; Smith and Hanawalt, 1969).

Recombination is also a dark mechanism of repair in which damage to the DNA while not being directly repaired, is either ignored or by-passed. This results in gaps in the daughter strands opposite the damage as DNA synthesis proceeds. The missing genetic information is supplied by information within the cell by means of recombination between the strands of DNA. Post-replication repair also is mediated by enzymes (Ganesan and Smith, 1968). Photoreactivation will be discussed in the next section.

Photoreactivation, Photoprotection, and Growth Delay

Photoreactivation may be defined as the reduction in the response of a biological system to UV irradiation with wavelengths between 220 and 300 nm by post-treatment with radiation at 310 to 549 nm. Photoreactivation was first noted as the reversal of the inactivation produced by UV radiation, however, it includes reversal of other effects of UV radiation such as mutation (Kelner, 1949b; Novick and Szilard, 1949; Newcombe and Whitehead, 1951; Kimball and Gaither, 1951) and reduce UV inhibition of growth (Blum et al., 1950; Blum and Matthews, 1952; Wells and Giese, 1950).

There are at the present time three types of photoreactivation. Type I photoreactivation or direct photoreactivation is mediated by an enzyme that splits thymine dimers induced in the DNA. The phenomenon was first reported in 1936 by Prat but the significance was not realized until 1949(a) by Kelner. Enzymatic photoreactivation requiring the phr+ gene is a nearly universal phenomenon. Exceptions are found among bacteria mutants and some mammalian cells. Also, neither sperm nor bacteriophage outside a host cell can be photoreactivated (Dulbecco, 1950; Hanawalt, 1969). Some of the more complicated cellular systems containing the enzyme are protozoa, unicellular green alge, frogs, reptiles, some fish, the Australian kangaroo, and other marsupials (Hanawalt, 1969).

The wavelengths which will photoreactivate inactivation differ for different organisms though the maximum is usually around 380 nm. The action spectrum for Streptomyces griseus shows a maximum at 430 nm (Kelner, 1951), while that of E. coli B/r shows three maxima at 324 nm, 350 nm, and 380 nm with 350 nm being the greatest (Jagger and Latarjet, 1956). The action spectrum for photoreactivation of T_2 phage corresponds to that of E. coli

(Kelner, 1951). The photoreactivation spectrum for Colpidium extends from 360 nm to 546 nm with no pronounced wavelength effect (Giese et al., 1953). These differences in the action spectra indicate that more than one type of photoreactivation chromophore may exist.

Much of the knowledge of direct photoreactivation comes from observation of in vitro photoreactivation of streptomycin resistance in transforming DNA of Hemophilus influenzae. The transforming DNA is not photoreactivable, either inside or outside the host cells or in extracts of the host cell. The host itself is not photoreactivable (Goodgal, et al., 1957). However, the transforming principle in extracts of E. coli B or baker's yeast is photoreactivable (Goodgal et al., 1957; Rupert, 1960). The active agent in the extracts is an enzyme as judged by susceptibility to proteolytic enzymes, heat lability, and the temperature dependence of the reaction. The purified extract of E. coli B contains a nondialyzable, heat labile fraction, a protein, and a dialyzable, heat stable fraction that may be considered a co-factor. The dialyzable component may be substituted by reduced NAD (Rupert, 1960). The yeast extract purified by a factor of 100 by normal protein purification procedures (Muhammed, 1966) only has a nondialyzable, heat labile fraction. Action spectra for the in vitro photoreactivation of transforming DNA have been obtained for both yeast and E. coli extracts (Setlow and Boling, 1963). The two spectra are similar with 350 and 385 nm being the most effective wavelengths. They have not provided an answer to the question of what the photon absorbing chromophore is since they do not correspond to a particular absorption spectrum. Flavin and pyridoxal have been suggested as possible chromophores for photoreactivation in the cells but there has not been any conclusive proof.

The second type of photoreactivation, type II or indirect, was discovered from observation of a mutant of E. coli B phr⁻ lacking the photoreactivating enzyme (Harm and Hillebrandt, 1962) and its parent strain. Both microorganisms may be photoreactivated but the phr⁻ strain does not split thymine dimers (Jagger and Stafford, 1965). The two strains differ in the temperature, dose, dose-rate, and wavelength dependencies for photoreactivation. Action spectra for photoreactivation of mutation to prototrophy in late log-phase cultures of E. coli H^3r30 which lack the photoreactivating enzyme is similar to those for photoprotection from killing in other strains and to the action spectrum for photoreactivation of killing in E. coli B phr⁻ with a maximum at 334 nm (Kondo and Jagger, 1966). The parent strains have action spectra similar to photoreactivation of killing in most other strains with a maximum at 385 nm. It is thought that indirect photoreactivation involves a delay in growth which allows more time for the damage to be repaired by the dark repair mechanisms. Photoprotection and delay in growth will be discussed later.

One other experiment must be mentioned that supports the theory of indirect photoreactivation. Cells of E. coli B phr⁺ and E. coli B phr⁻ are labeled with tritiated thymidine and then exposed to inactivating UV at 254 nm. These cells are immediately exposed to visible light at various wavelengths and assayed for thymine containing dimers. The phr⁻ strain is unable to split thymine dimers and no photoreactivation is observed after illumination at 405 nm. The amount of splitting of thymine dimers in the phr⁺ strain after photoreactivation at 334 nm is only 45 percent of the amount of splitting observed at 405 nm for the same amount of biological photoreactivation. The conclusion is that all of the photoreactivation of the phr⁻ and part of the photoreactivation of the phr⁺ at 334 nm is indirect and is not due to thymine dimer splitting (Jagger et al., 1969). Further support of the hypothesis that

indirect photoreactivation results from enhancement of dark repair is obtained from the action spectrum of E. coli B_{s-1} which lacks the dark repair ability. Very little photoreactivation is observed at 334 nm.

The third type of photoreactivation has only recently been observed. Action spectra of Staphylococcus epidermidis (Ikenaga et al., 1970) and of Streptomyces griseus (Jagger et al., 1970) show a secondary peak at 313 nm which has not been reported previously. This peak is in addition to the one at 436 nm which involves the enzymatic photoreactivation of lethality. Type III photoreactivation has little or no dependence upon temperature and dose-rate so it probably is not enzymatic. The mechanism of type III photoreactivation involves the removal of uracil-thymine heteroadducts. (Ikenaga et al., 1970).

Photoprotection was observed almost simultaneously by Weatherwax (1956) in America who found that E. coli B and not E. coli B/r showed the effect and by Miki (1956) in Japan who found photoprotection in E. coli K12 (λ) but not in E. coli K12S. Photoprotection is a phenomenon in which cells have a greater ability to survive if subjected to radiation at wavelengths between 310 and 370 nm before irradiation (Jagger and Stafford, 1962). Photoprotection is independent of temperature and is a purely photochemical reaction, differing from type I photoreactivation which shows definite temperature and dose-rate effects and involves enzymes. Photoprotection is a far less general phenomenon than photoreactivation. Its occurrence among microorganisms and protozoa has been found to be diverse.

Action spectra studies for photoprotection in E. coli and P. aeruginosa show that the phenomenon is dependent on wavelengths from 330 to 380 nm peaking at 334 nm (Jagger and Stafford, 1962). The fact that both organisms have action spectra with a similar smooth curve and with a single peak suggests

that a single chromophore is involved. It has been suggested that at 334 nm nucleic acids could be absorbing the radiation but the transforming principle of H. influenzae (pure DNA) fails to show photoprotection as does intracellular phage T_2. NADH whose absorption spectrum peaks at 340 nm has been considered a likely candidate.

Action spectra for both photoprotection and indirect photoreactivation show a maximum efficiency at 334 nm. It is thought that both induce delay in growth which allows excision repair to operate on the nucleic acid damage. To prove the theory an action spectrum for growth delay of E. coli B in nutrient broth has been obtained (Jagger et al., 1964). It shows a single peak at 338 nm and is indistinguishable from the action spectra for photoprotection and indirect photoreactivation. Microscopic observation show that the growth delay is accompanied by division delay. It has been stated previously that the action spectra coincides with the absorption spectrum of NADH, however, Kashket and Brodie (1963) have shown that nonlethal doses of polychromatic near-UV can block aerobic pathways for energy metabolism in E. coli B and P. aeruginosa. Irradiated E. coli shows a drastic loss of benzoquinone Q_8 and a naphthoquinone C_{45}. Cell free extracts show that the electron transport chain from NADH to oxygen and from succinate to oxygen can be inactivated by near-UV and subsequently restored by addition of quinones. Furthermore, the action spectra for induction of growth delay (Jagger et al., 1964) and division delay (Phillips et al., 1967) in E. coli B are similar to the absorption spectrum for destruction of vitamin K_2 in isooctane and to the action spectrum for destruction of vitamin K_2 in isooctane (Jagger and Takebe, 1968). These findings suggest that isoprenoid quinones are the chromophore absorbing the energy at 334 nm.

Growth delay can also be induced by far-UV light. To see if quinones are the chromophores at these wavelengths an action spectrum was obtained for growth

delay induced in E. coli B/r. It resembles the action spectrum for killing obtained in the same experiments and suggests that nucleic acids are the chromophore in growth delay induced by wavelengths below 330 nm. Isoprenoid quinones play a negligible role (Takebe and Jagger, 1969).

Recombinationless Mutants

Since this investigation is on the action spectrum for lethality in a recA mutant, the properties of recombinationless mutants will be discussed briefly. Bacterial mutants that are defective in recombination were recently isolated and partially characterized by Clark (1967). Mapping studies of E. coli and S. typhimurium show that at least three genes recA, recB, and recC must function in order for the cell to undergo maximum recombination. These three loci lie within a 5 percent segment of the chromosome (Figure 1).

RecB and recC code for an ATP-dependent nuclease which has both double-stranded exonucleolytic activity (Buttin and Wright, 1968; Oishi, 1969; Barbour and Clark, 1970) and a single-strand endonucleolytic activity (Goldmark and Linn, 1970). A mutation at either the recB or recC loci will eliminate nuclease activity. At the present time the molecular nature of the recA product is unknown. The recA gene product does seem to control or inhibit the recB recC nuclease activity in vivo. In the absence of a functional recA product the recB recC nuclease contributes to extensive DNA degradation. The recA gene product appears to be absolutely necessary for genetic recombination. Recombination frequencies in recA mutants are about 10^{-3} to 10^{-5} compared to a value of 1 for rec+ cells (Willetts and Mount, 1969). The recB and recC mutants show an intermediate level of recombination.

Recombinationless mutants are also extremely sensitive to UV, X-rays, and chemical mutagens. The recA mutants are the most sensitive while the recB and

recC mutants show intermediate sensitivities. The recA mutants cannot perform post-replication repair after UV irradiation while recB and recC mutants show no deficiency in this ability.

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Figure 1

Mutations in *E. coli* affecting radiation sensitivity. (Smith, 1970)

Mutant Designation	Map Position	Deficiency or Sensitivity
exr (lex)	(79)	UV; X-rays
lon	11	filament formation
phr	(17)	photoreactivation
polA1	75	DNA polymerase; X-rays
ras	(11)	UV
recA	51.7	recombination, UV, X-ray
recB	54.2	recombination, UV, X-ray
recC	54.5	recombination, UV, X-ray
ref II	(88)	UV sensitive, colicin E2 resistant, recombination (?)
res	(75)	UV; X-ray; DNA polymerase
uvrA	80	UV
uvrB	18	UV
uvrC	27	UV
uvrD	74	UV

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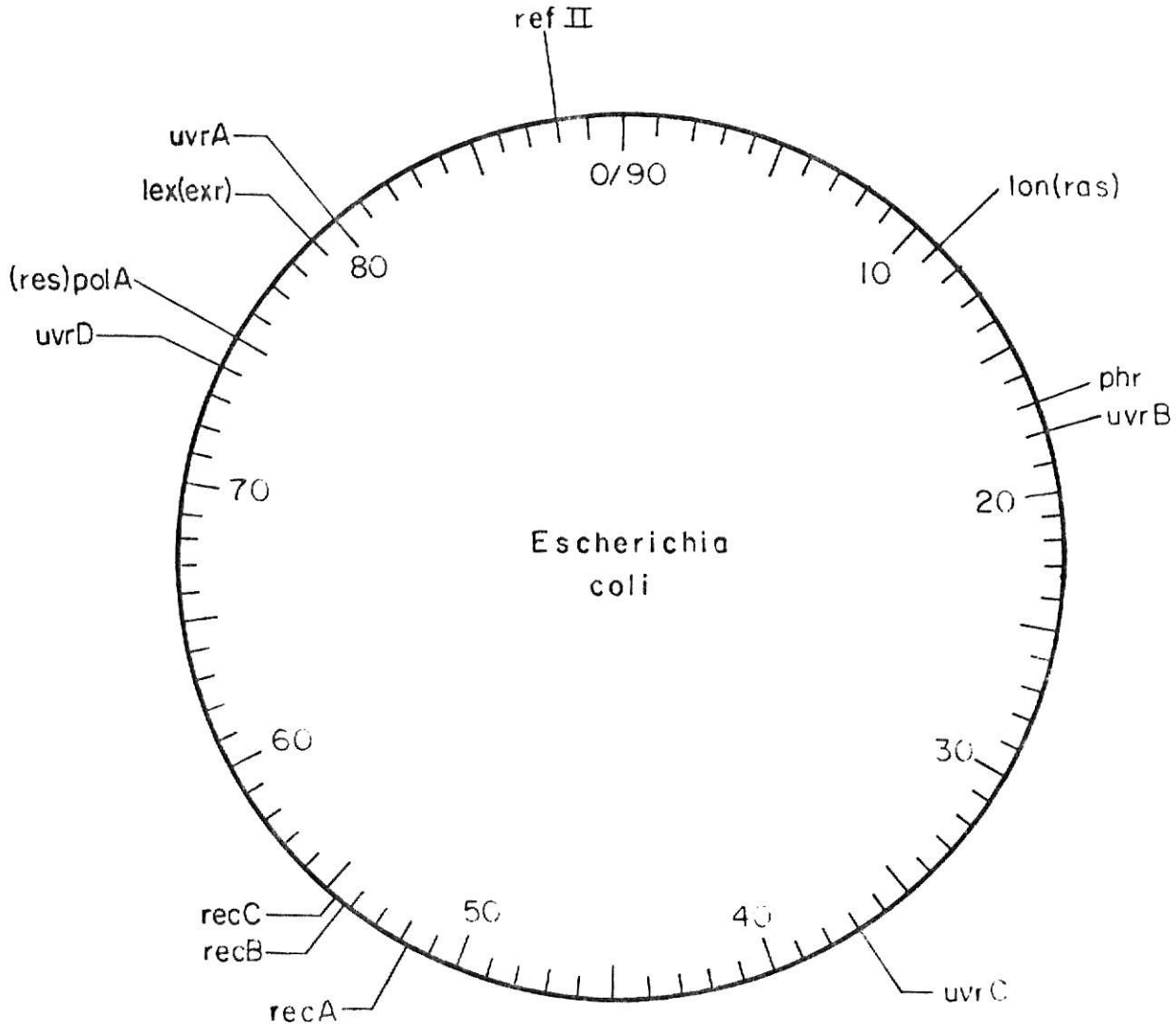

ref II
0/90
lon(ras)
10
phr
uvrB
20
30
uvrC
40
50
recA
recB
recC
60
70
(res)polA
uvrD
lex(exr)
uvrA
80
Escherichia
coli

Figure 1

MATERIALS AND METHODS

Bacterial Strains

S. typhimurium KSU 2480, a mutant of LT2, that requires tryptophan and E. coli K12 AB2463 that requires threonine, leucine, histidine, proline, arginine, and thiamine were used in the experiments. Both strains are recA mutants and are light sensitive. Wildtype strains were used as controls.

Media

Nutrient agar was prepared by mxing 18.5 grams of dehydrated nutrient agar (Difco Laboratories, Detroit, Michigan) in 800 ml of deionized water.

M-9 sodium phosphate buffer at pH of 7.0 was prepared by mixing 10 ml of 100X-minimal salts solution, 40 ml of 25X-minimal salts solution, and 950 ml of water. One hundredX-minimal salts solution contains the following salts in grams per liter of deionized water: NH_4Cl, 100; and $MgSO_4 \cdot 7H_2O$, 20. Twenty-fiveX-minimal salts solution contains the following salts in grams per liter of deionized water: KH_2PO_4, 75; $Na_2HPO_4 \cdot 7H_2O$, 150; and NaCl, 125 (Mallinckroodt Chemical Works, St. Louis, Missouri).

Amino acids used were tryptophan, threonine, leucine, histidine, proline, and arginine at a concentration of 10 mg/l of deionized H_2O. Tryptophan at a concentration of 2 mg/l of deionized H_2O was also used. Thiamine was similarly used at a concentration of 1 mg/l of deionized H_2O. The vitamin and amino acids were purchased from Mallinckroodt Chemical Works, St. Louis, Missouri.

To check the absorption of the medium and thus be assured that an appreciable amount of the energy was not being absorbed by the amino acids the absorbance was taken at various wavelengths using the Zeiss QM II (Table 1).

Table 1. Absorbance of the medium.

Wavelength (nm)	M-9 + 2 mg/l tryptophan	M-9 + 5 mg/l tryptophan	M-9 + 10 mg/l tryptophan	M-9 + 10 mg/l threonine, leucine histidine, proline, arginine + 2 mg/l thiamine
	Absorbance			
240	.068	.092	.135	.115
250	.068	.108	.155	.086
254	.071	.130	.185	.082
260	.080	.170	.240	.087
270	.095	.230	.335	.076
280	.097	.246	.345	.060
290	.056	.185	.243	.034
300	.008	.033	.060	.018
310	.002	.008	.028	.010

Irradiation Sources

Radiant energy was supplied by five sources which varied in the irradiance which is the power striking the object per unit area of the object (Jagger, 1967) and in the purity of the beam.

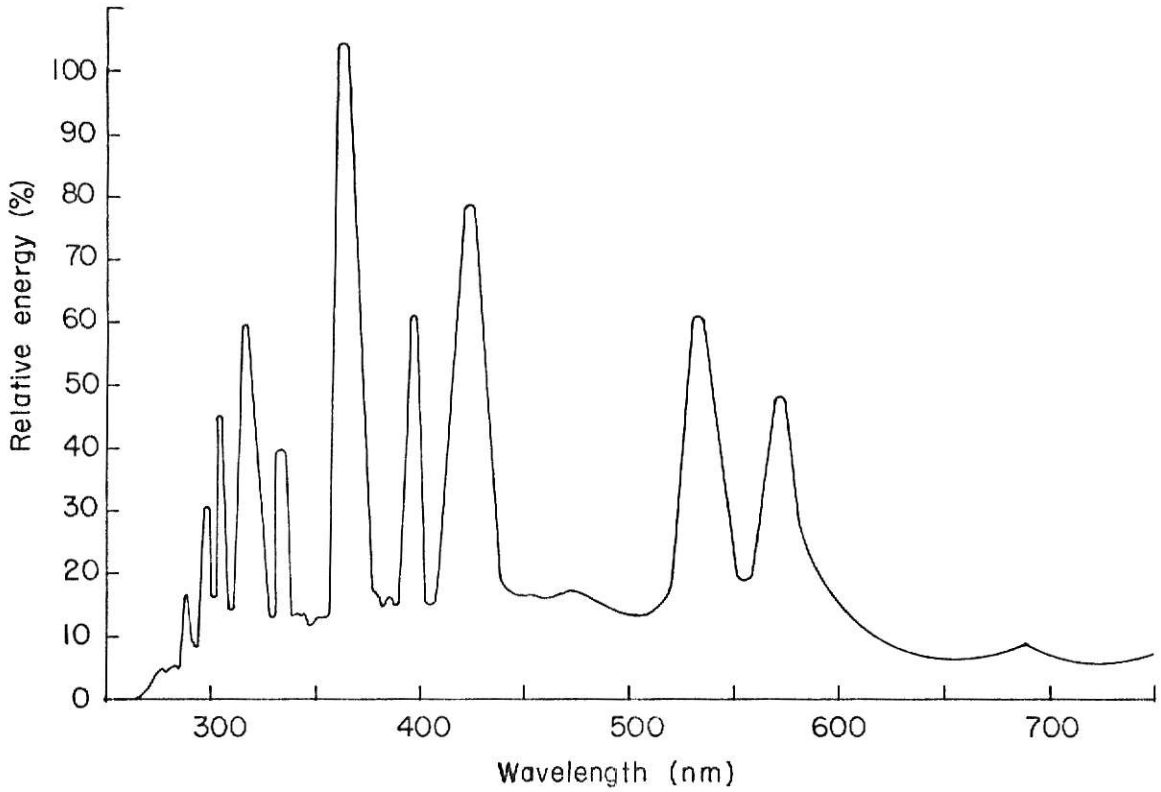
For UV (254 nm) a low pressure mercury lamp (Penray SC-11) with a filter to reduce the long UV and visible components was used. More than 95% of its emission was at 254 nm (Webb and Lorenz, 1970). A Bausch and Lomb high intensity monochromator (No. 33-86-26-07) with a 200 W super pressure mercury vapor arc lamp (Osram HBO) was used at the wavelengths 313 and 365 nm. For wavelengths in the far-UV and the near-UV (240-365 nm) a Bausch and Lomb high intensity monochromator with a xenon arc lamp (Osram HBO) was used. Spectral output for the xenon arc lamp and the super pressure mercury arc lamp is seen in Figure 2. Energy for the visible wavelengths of 460 and 550 nm was produced

Figure 2

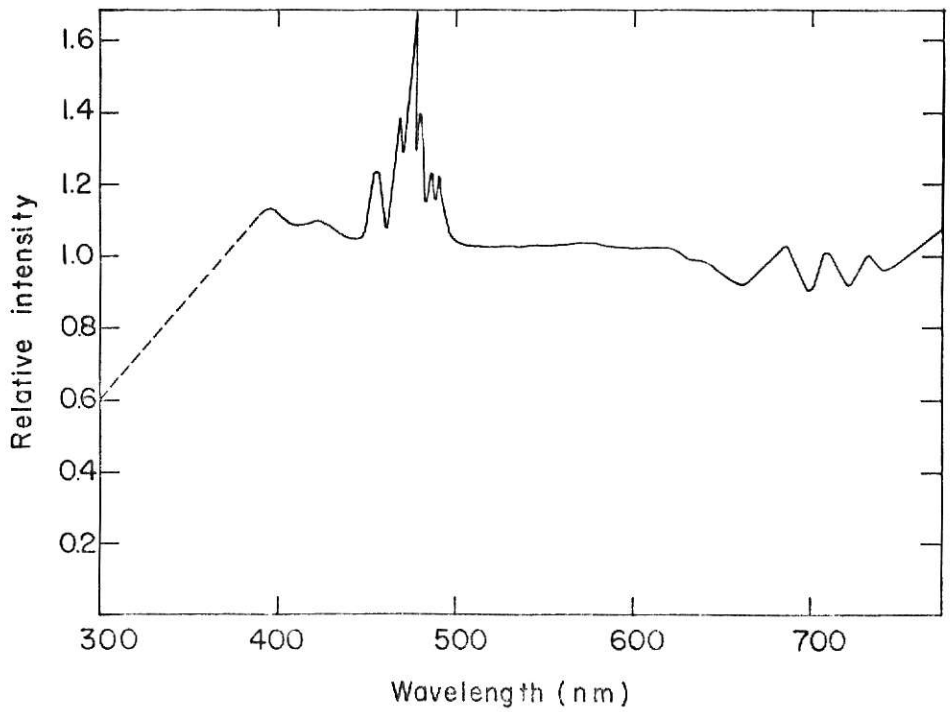
Transmission Spectra of Lamps

(a) Spectral output of super pressure mercury arc lamp (Osram HBO 200w).

(b) Spectral output of xenon arc lamp (Osram GMBH Kommandit-gesellschaft).


Relative energy (%)
100
90
80
70
60
50
40
30
20
10
0
300
400
500
600
700
Wavelength (nm)

(a)


Relative intensity
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
300
400
500
600
700
Wavelength (nm)

(b)

Figure 2

by the General Electric Marc 300 lamp in a specially designed housing. Heat filters were used at these wavelengths. For wavelengths between 313 and 365 nm the Schoeffel high intensity monochromator was also used. The components are: Bausch and Lomb 500 nm monochromator; a lamp housing, Schoeffel LH152N; a lamp, Hanovia 975C 2500 W xenon arc; a power supply, Schoeffel LPS400; a starter, Schoeffel LPS400S, 200 ampere direct current starter; and a pre-dispersion prism.

Interference filters purchased from Baird-Atomic were used to insure specificity of wavelengths at 313, 350, 365, 460, and 550 nm. A Corning 0140 filter was used at wavelengths of 330 and 340 nm. The percent of transmission in arbitrary units of these filters using a Cary spectrophotometer is given in Figure 3. Irradiances below 100 erg/mm^2/sec were measured with a Schwarz vacuum thermopile calibrated against a National Bureau of Standards lamp. The output of the thermopile was measured with a Keithley 150 B microvoltammeter. Irradiances above 100 erg/mm^2/sec were measured with a YSI-Kettering Radiometer (Model 65), secondarily standardized with reference to the Schwarz thermopile.

Preparation of Cultures for Irradiation

For the inactivation studies of stationary cultures, a clone was selected and then streaked on a nutrient agar plate. This was followed by incubation at 37° C in the dark for two days to ensure a population of stationary phase cells. A loop of surface growth was diluted in M-9 salts and placed on a vortex shaker. The suspension was diluted to give a cell concentration of about 10^7 cells per ml for the irradiation.

For the inactivation of log phase cultures of S. typhimurium, cells were diluted into tubes containing M-9 salts plus 400 mg/l glucose plus 10mg/l tryptophan and incubated at 37° C in the dark for 15-18 hours. The cells were then diluted

Figure 3

Transmission Spectra of Interference Filters (Baird-Atomic) and Cut-off Filter 0140 (Corning)

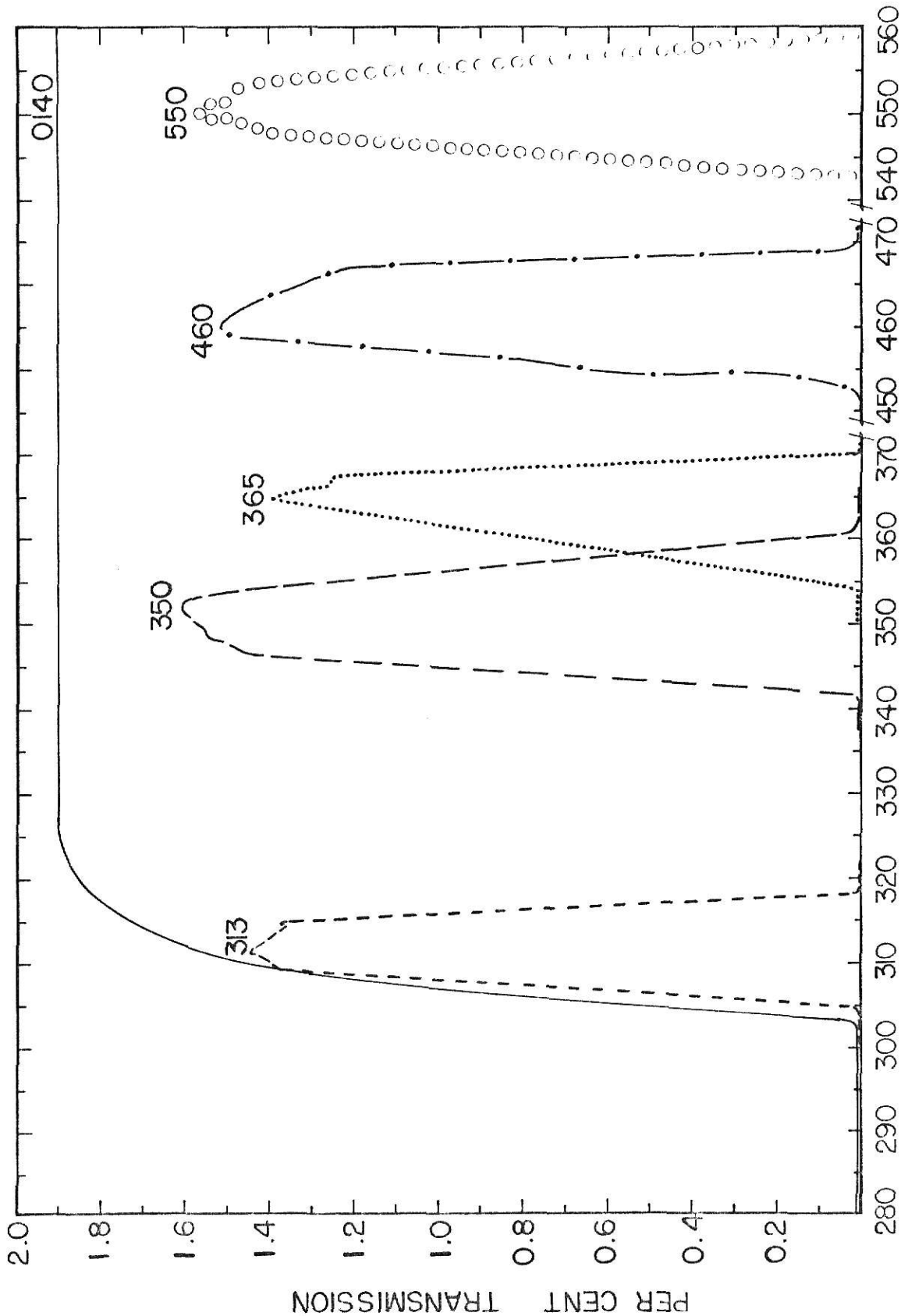

0140
550
460
365
350
313
PER CENT TRANSMISSION
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
280 290 300 310 320 330 340 350 360 370 450 460 470 540 550 560
WAVELENGTH nm

Figure 3

1/100 into M-9 salts plus 400 mg/l glucose plus 2 mg/l tryptophan and incubated at 37° C in the dark for 3-3 1/2 hours. These cultures which had a concentration of about 10^7 cells per ml were used for the irradiation. This density insured that at most wavelengths all radiation, direct and scattered was absorbed.

To insure that cells were in log phase at three hours after a 1/100 dilution of the overnight culture, the following experiment was done. Stationary cells of S. typhimurium taken from a nutrient agar plate were diluted into M-9 to a concentration of $1X10^8$. The cells were then diluted 1/20 into M-9 plus 2 mg/l tryptophan plus 400 mg/l glucose and incubated at 37° C in the dark. Two milliliter samples were taken every hour. One milliliter of cells was irradiated at 313 nm using the super pressure mercury lamp for fifteen minutes with a dose of 150 erg/mm^2/sec (EMS). The other milliliter of cells was irradiated at 254 nm using the low pressure mercury lamp for six seconds at a dose of 6.55 EMS. After 4, 6, and 8 hours of growth the cells had to be diluted to a concentration of not greater than $1X10^8$ for irradiation. Before irradiation at either wavelength a sample was taken at T=0 to serve as a control. The survival after irradiation was divided by the control to obtain the percent survival at each hour as shown in Figure 4. Cells were in log phase after three hours.

For the inactivation of log cultures of E. coli K12 AB 2463, the procedure was the same except the M-9 salts contained 400 mg/l glucose plus 10 mg/l of threonine, leucine, histidine, proline, arginine, and 1 mg/l thiamine.

Generation Time at Room Temperature

Since log cells were irradiated in supplemented minimal media, the generation time at room temperature had to be determined so that the proper corrections could be made for exposures of long duration. Log cells of S.

typhimurium at a concentration of $1X10^7$, grown as explained above, were placed in an identical set up at room temperature as those cells normally irradiated except these cells were not irradiated. At various times for 6 hours aliquots were plated on nutrient agar and a growth curve seen in Figure 5 was obtained. The increase in 6 hours was only about 2.5 fold. This slight increase would not affect the results significantly.

Conditions for Irradiation

Irradiation vessels were quartz tubes for irradiation with far-UV (200-300 nm) or pyrex tubes for irradiation about 300 nm with an inside diameter of 1 cm. The percentage of light transmitted in arbitrary units by the pyrex cuvette at the various wavelengths is seen in Figure 6. Bubbling air through a capillary tube fitted in the bottom of the vessels provided a means of stirring the suspension. Aliquots of 0.2 ml of the appropriately diluted samples taken at various times were spread on nutrient agar plates. Dilutions were made in M-9 salts. The plates were incubated in the dark at 37° C for 24 hours for the S. typhimurium and for 48 hours for the E. coli. Survival was defined as colony-forming ability. Temperature of the cell suspensions was maintained at 24-26° C during all irradiations except those specified at o° C.

All work was done under light from Champion F 40/GO gold fluorescent bulbs which only emit wavelengths above 500 nm to prevent inactivation from fluorescent room lights.

Figure 4

Evidence that an overnight culture of Salmonella typhimurium KSU 2480 diluted 1/100 is in logarithmic phase after three hours growth at 37° C.

Figure 5

Survival curve of Salmonella typhimurium KSU 2480 at 25° C without irradiation.

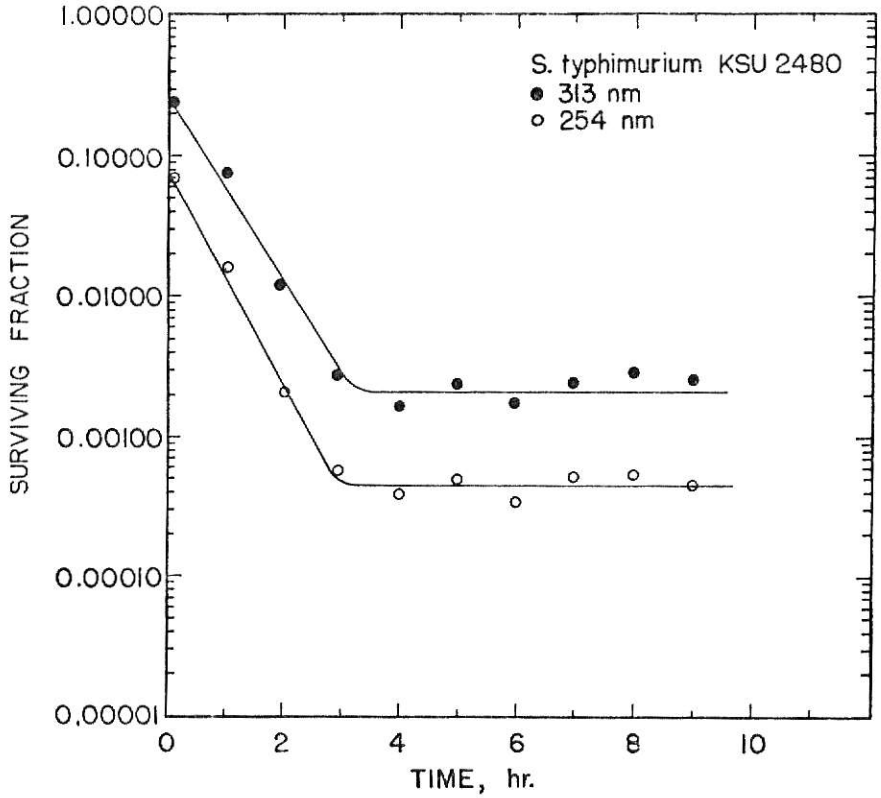


Figure 4

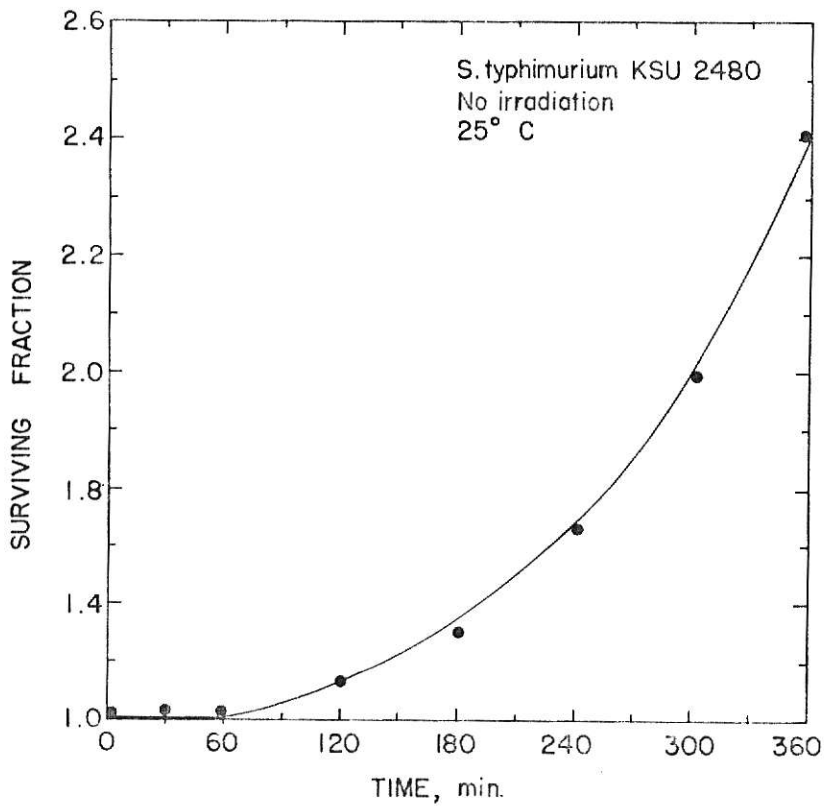


Figure 5

Figure 6

Transmission Spectrum of the Pyrex Cuvette.

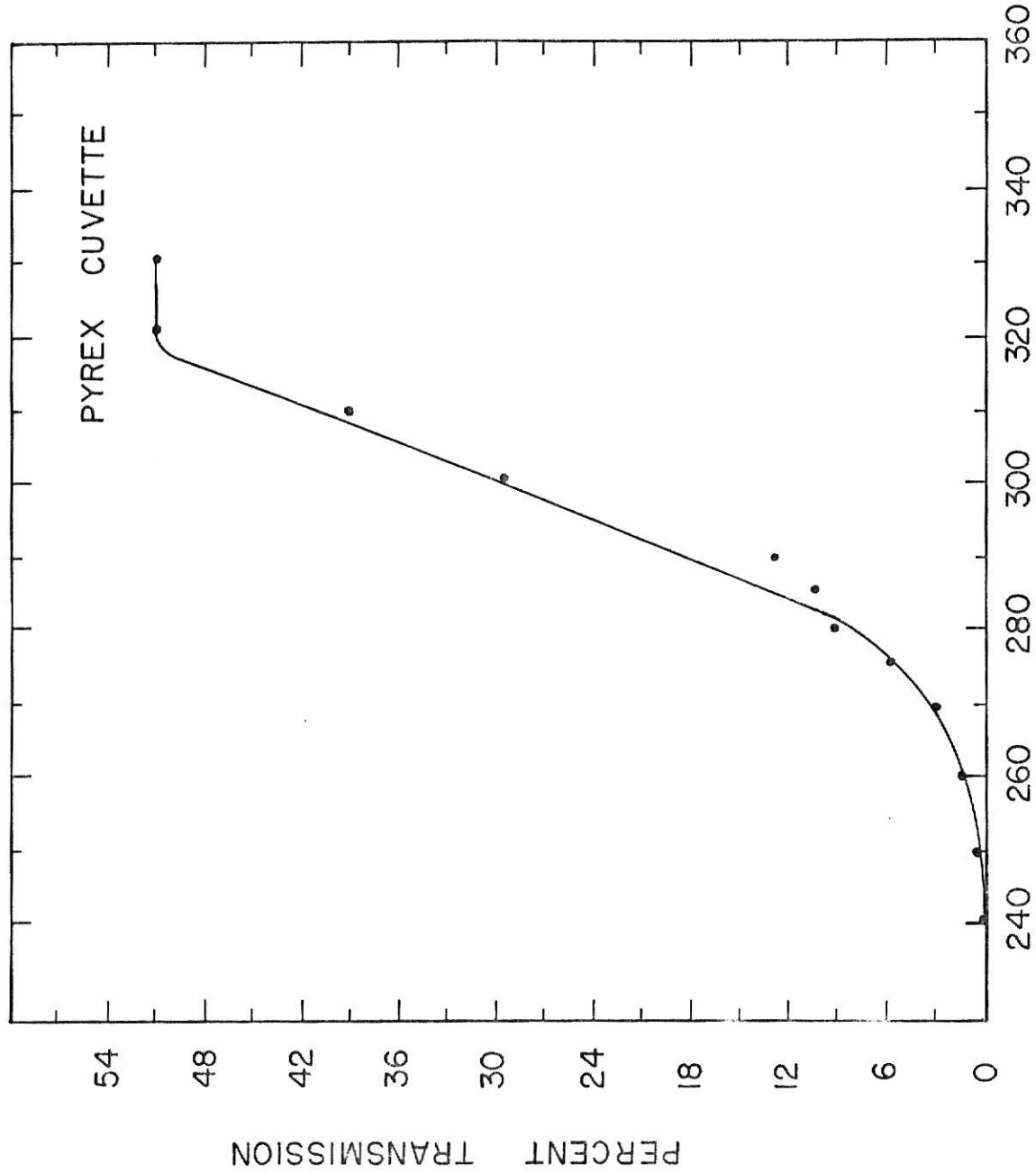

PYREX CUVETTE
PERCENT TRANSMISSION
0
6
12
18
24
30
36
42
48
54
240
260
280
300
320
340
360
WAVELENGTH, nm

Figure 6

RESULTS

Dose-Response Relationship

As previously stated, the purpose of this investigation was to obtain an action spectrum of the lethality of a recombinationless strain of S. typhimurium using monochromatic light. A series of inactivation curves were collected for both exponential and stationary phase cells. A lethality of three cycles of kill was found to be optimal due to the fact that lethality beyond three logs of kill involves such a small fraction of the total population of cells. At some wavelengths, however, energy from the various light sources was sufficient to allow as much as five cycles of kill, while at other wavelengths the energy output was not sufficient to obtain even three cycles of kill. In all the experiments at all wavelengths used to plot an action spectrum at least one cycle of kill was obtained.

At wavelengths between 240 and 365 nm sufficient energy was available so that short exposures in seconds to a maximum of 2 hours could be employed. At 460 and 550 nm insufficient energy was available for short exposures and therefore the irradiation period was extended to 5 hours. Because of the possibility that growth of the log cells during irradiation periods of long duration in the presence of a supplemented minimal medium might occur, the experiment as stated in the Materials and Methods was performed. During this time the cell population increased only about 2.0 fold, therefore no correction was made for the cells irradiated at these wavelengths.

In all experiments the total dose used was not sufficient to inactivate wildtype S. typhimurium or E. coli.

Inactivation Using the Bausch and Lomb Monochromator with a Xenon Arc Lamp

The xenon arc lamp in a Bausch and Lomb monochromator was used for wavelengths between 240 and 365 nm without any filters other than the cuvette. Above 310 nm there was not sufficient energy to inactivate 90% of the stationary cells within the time allotted for exposure. Inactivation of an *E. coli recA* was obtained for wavelengths up to 300 nm for comparison with the *S. typhimurium recA*. Curves at these wavelengths are shown in Figures 9-28. A low pressure mercury lamp was used at 254 nm seen in Figures 7 and 8.

The inactivation curves are not linear exponentials representing one hit-inactivation except for at least 10% survival in all curves. The curve shape always has two parts: 1) an initial exponential; 2) a tailing effect with the slope decreasing at the higher doses. The question arose as to the possibility of a mixed population both of which are single-hits, one sensitive and representing at least 90% of the cells, the other resistant and representing 10% of the cells. Isolation of a single colony from the more resistant part of the curve was made and the experiment repeated. If this heterogeneity were due to genetic differences then reirradiation should yield a simple survival curve with a slope characteristic of the more resistant species in the original population. Heterogeneity in survival can also represent phenotypic difference within a population of genetically identical cells. Those cells at a particular stage in the division cycle may be more sensitive than those at another stage in the cycle (Smith and Hanawalt, 1969). This is not the case in these experiments since inactivation of stationary and log cultures gave the same curve shape. One possible explanation is that this heterogeneity could be induced by the irradiation itself. Nonlethal events in a system could somehow stimulate the development of resistance to further

events. This typical curve shape has been reported previously for recA mutants (Howard-Flanders and Boyce, 1966).

Figure 7

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 254 nm using a low pressure mercury lamp (Penray SC-11) with a filter to reduce the long and visible components.

Figure 8

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 254 nm using a low pressure mercury lamp (Penray SC-11) with a filter to reduce the long and visible components.

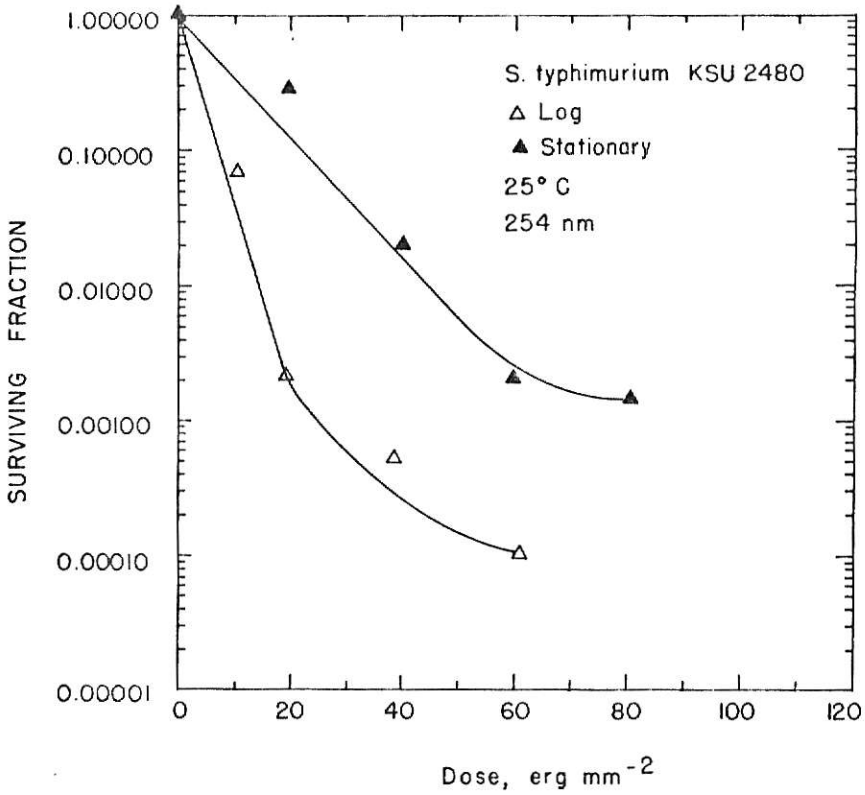


Figure 7

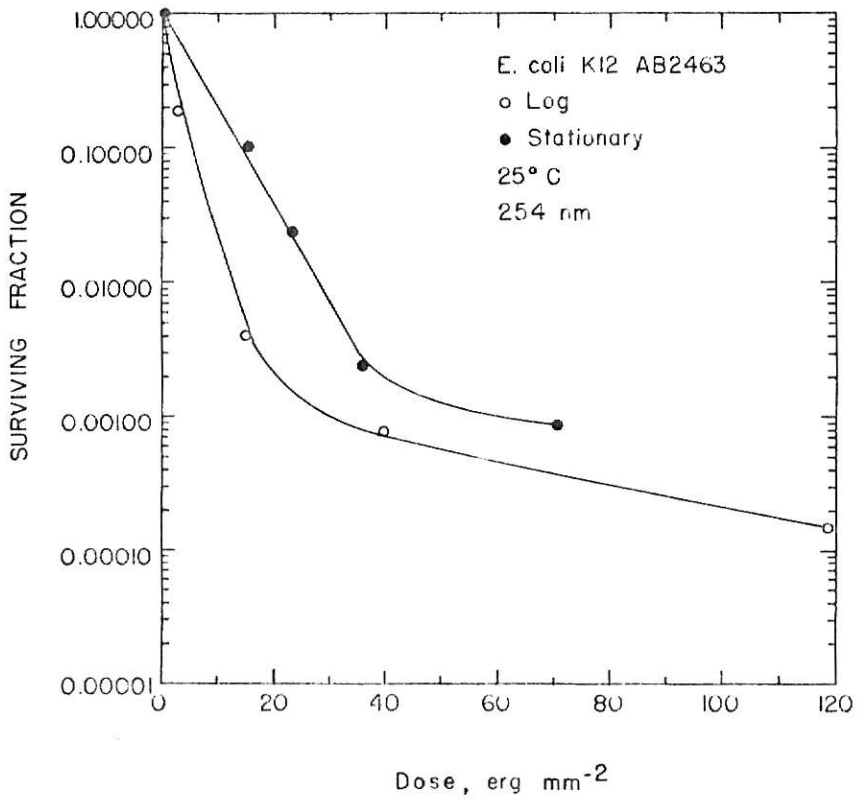


Figure 8

Figure 9

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 240 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 10

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 240 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

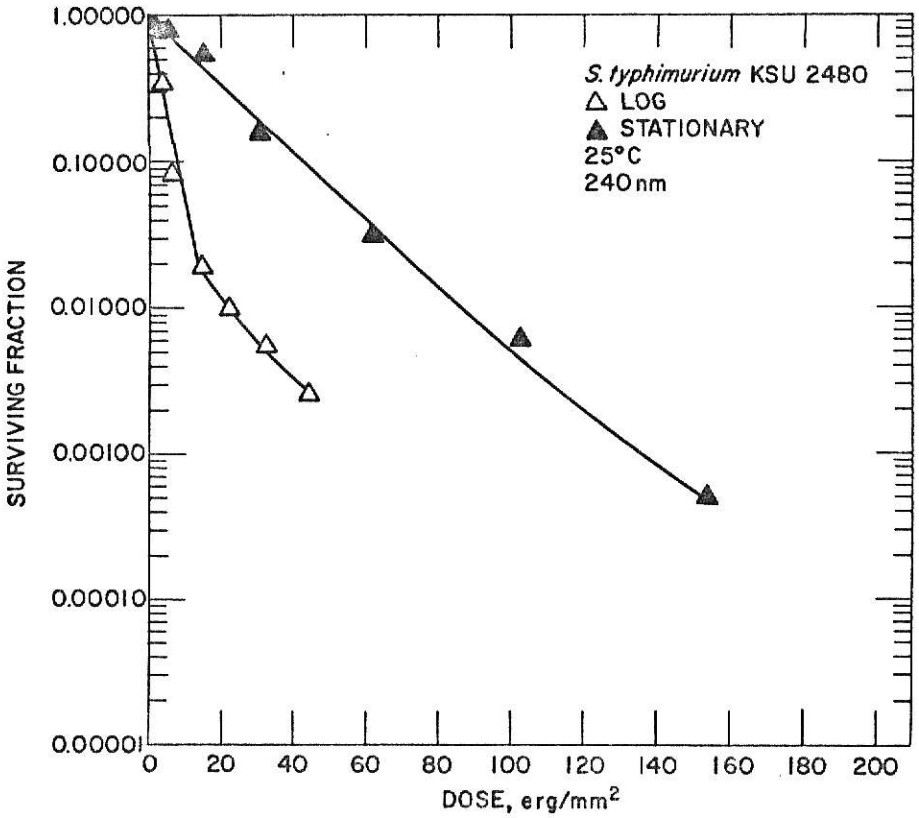


Figure 9

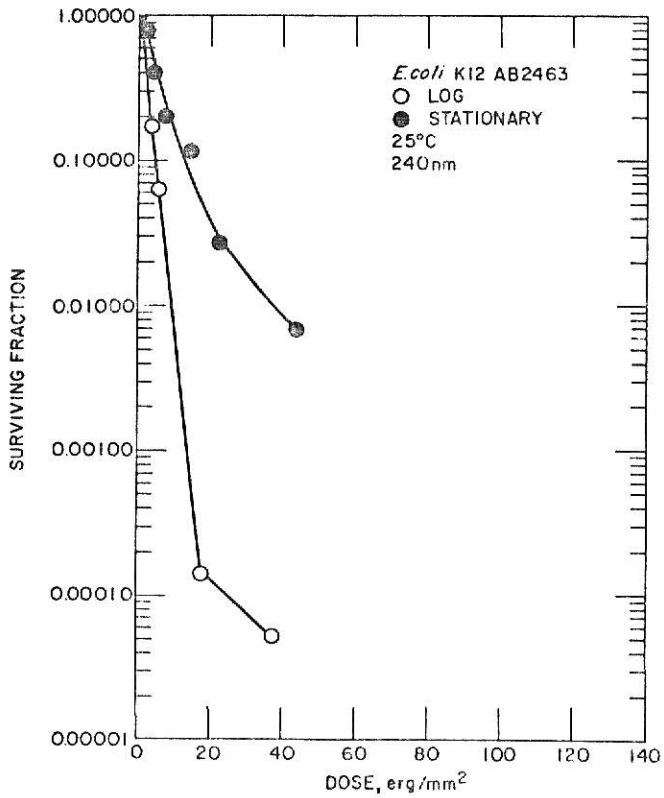


Figure 10

Figure 11

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 250 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 12

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 250 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

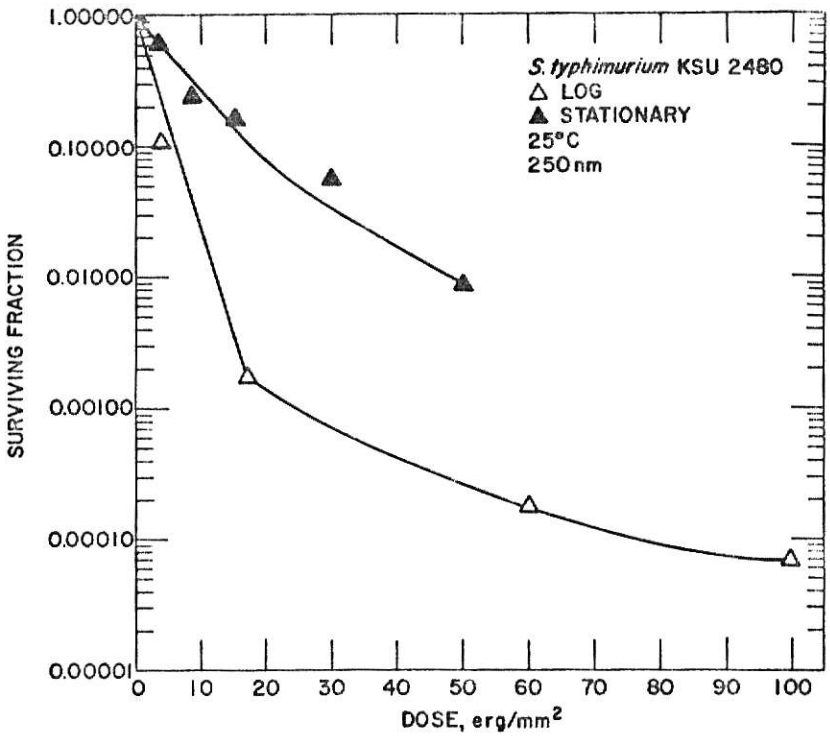


Figure 11

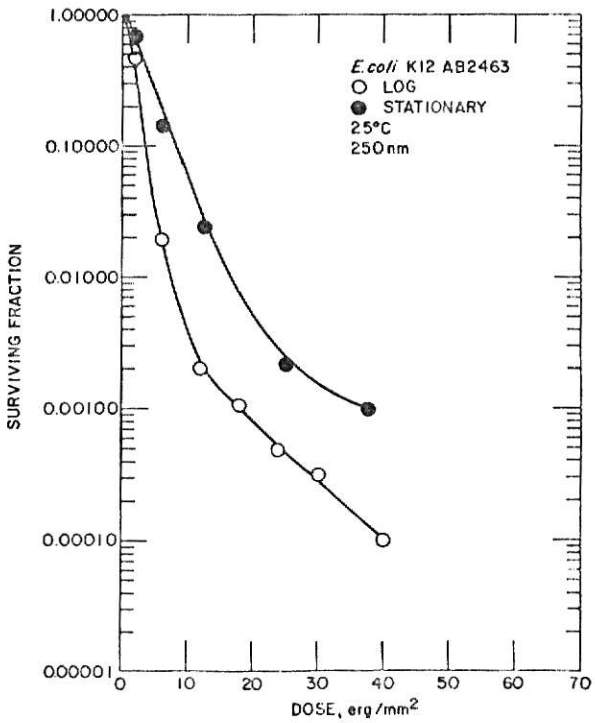


Figure 12

Figure 13

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 260 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 14

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 260 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

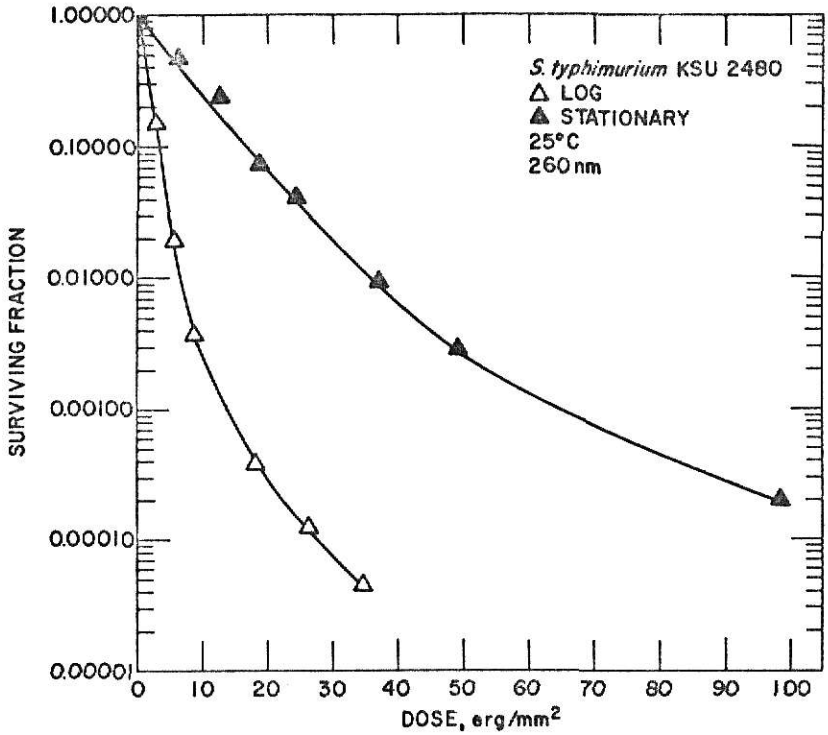


Figure 13

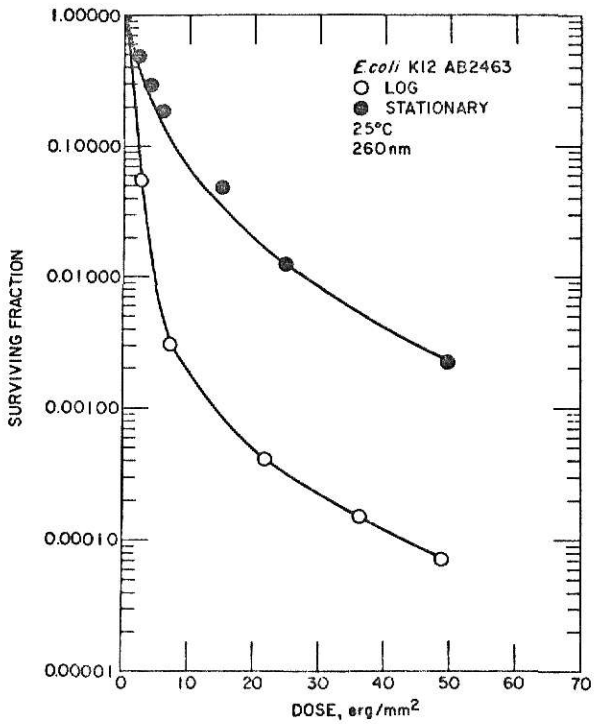


Figure 14

Figure 15

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 270 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 16

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 270 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

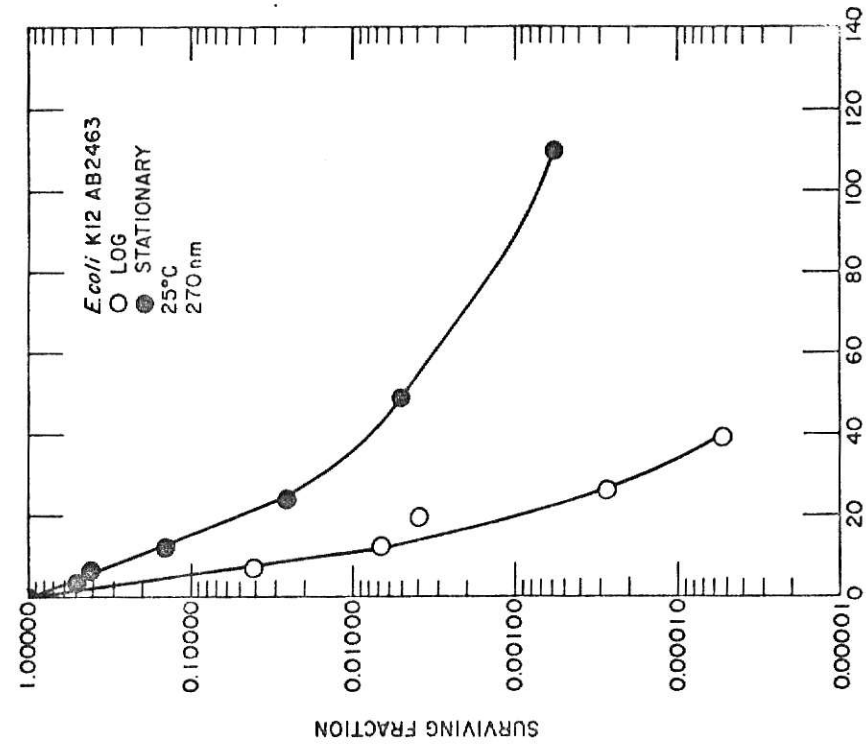


Figure 16

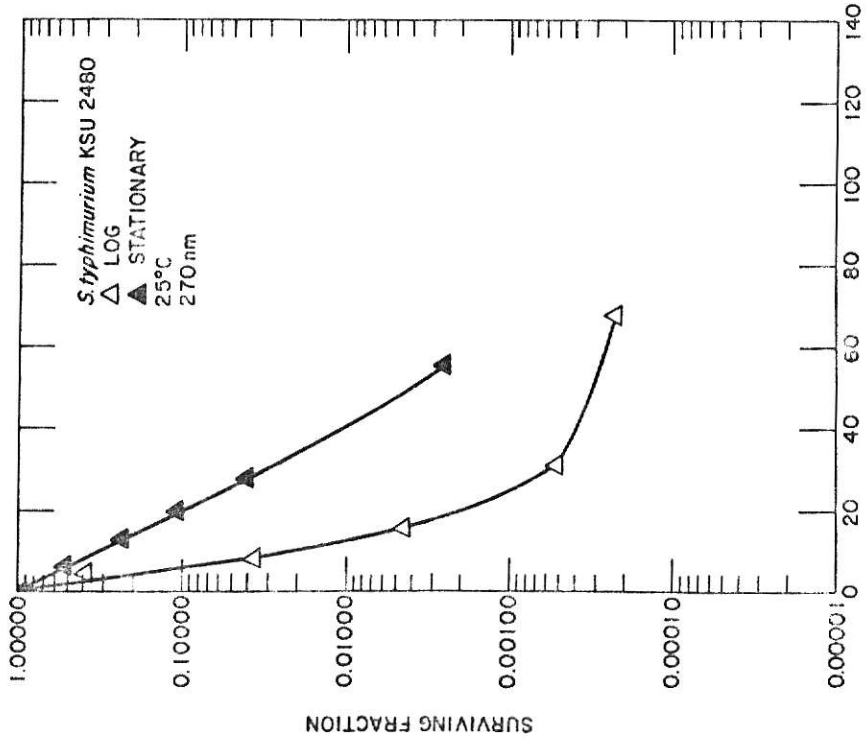


Figure 15

Figure 17

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 280 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 18

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 280 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

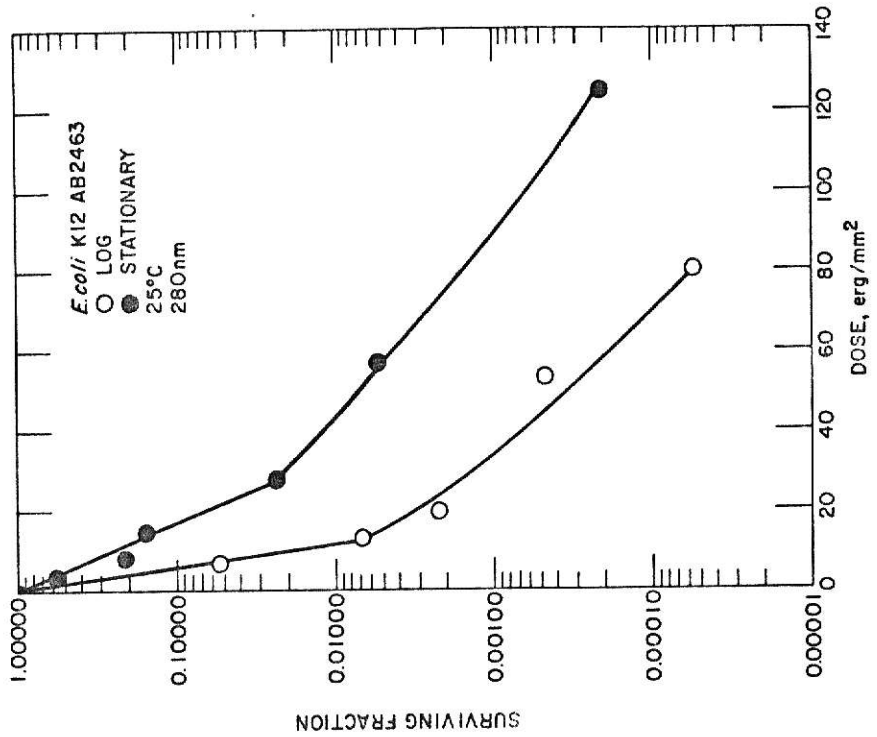


Figure 18

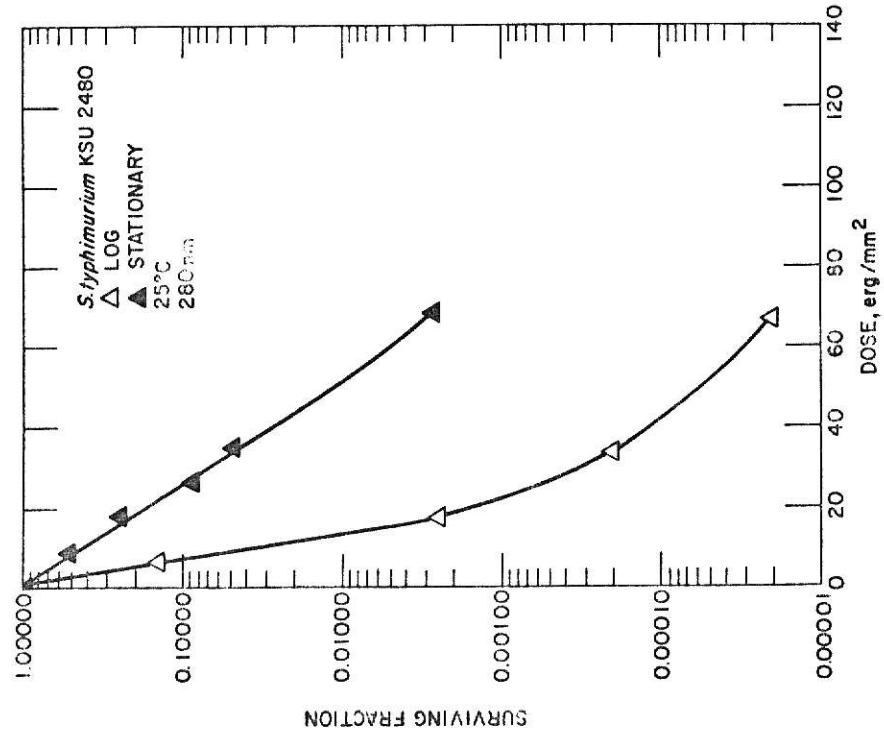


Figure 17

Figure 19

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 290 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 20

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 290 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

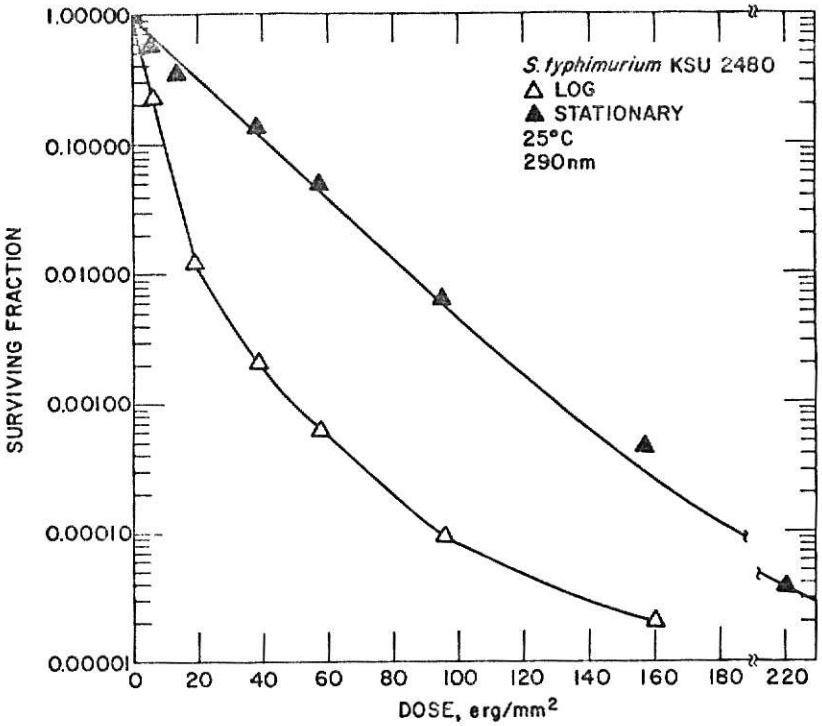


Figure 19

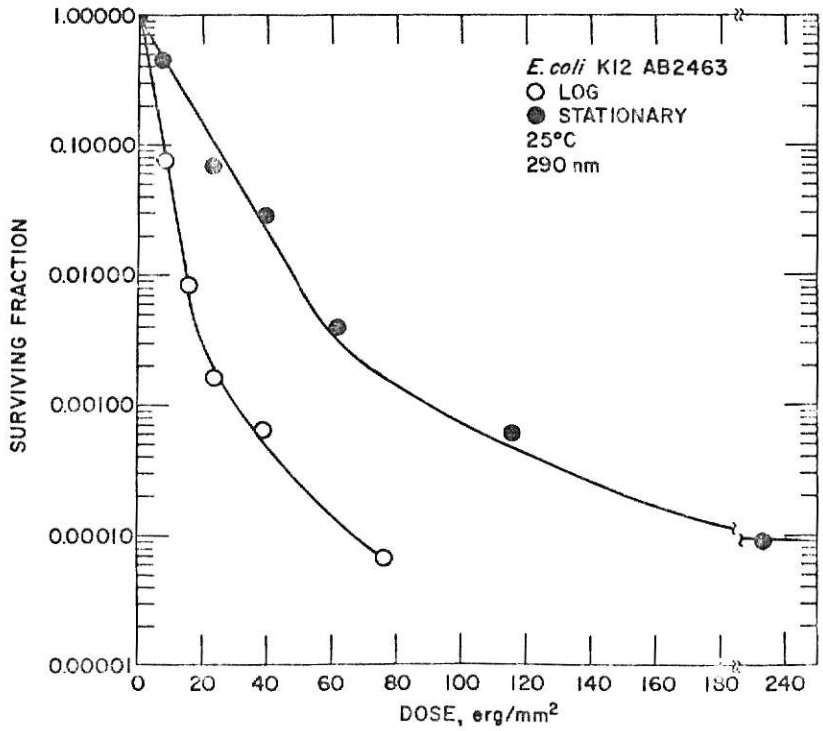


Figure 20

Figure 21

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 300 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 22

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 300 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

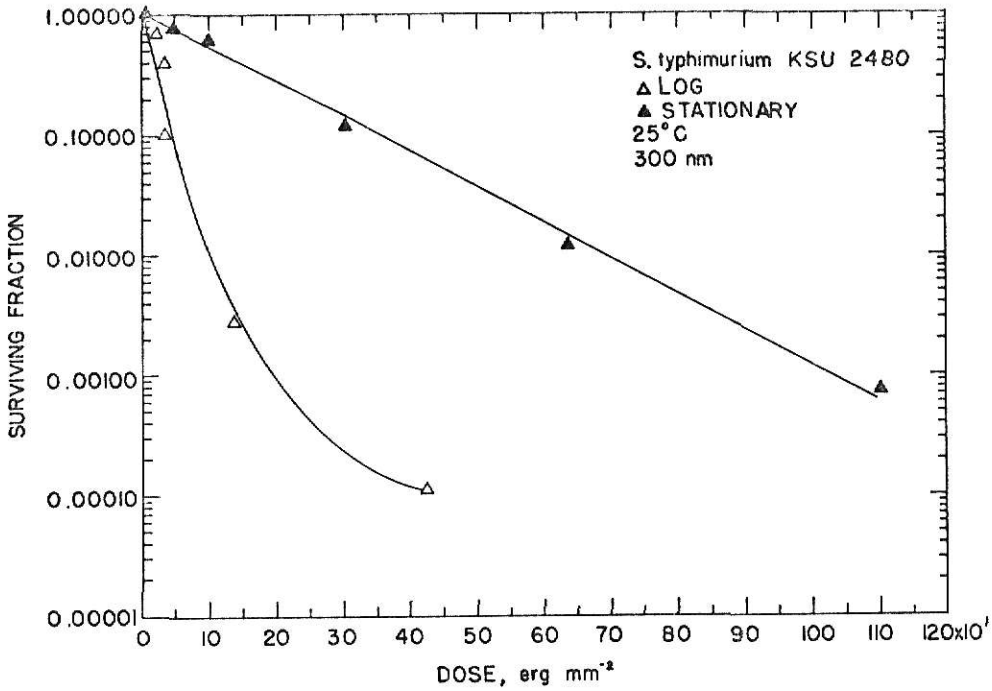


Figure 21

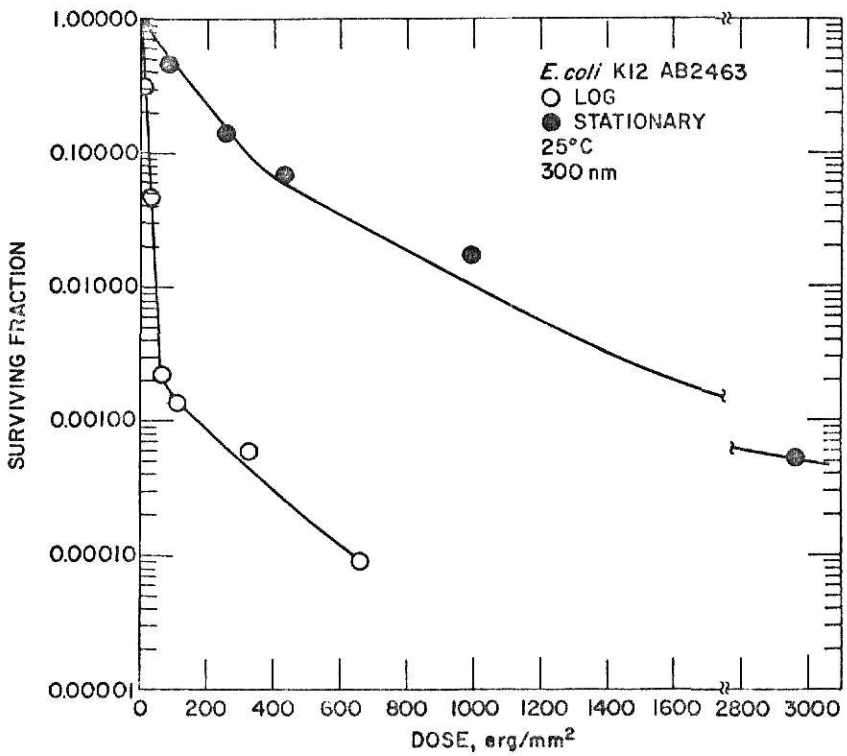


Figure 22

Figure 23

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 306 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 24

Inactivation curves of log phase cultures of Salmonella typhimurium KSU 2480 at 313 nm using a Bausch and Lomb monochromator with a xenon arc lamp and a Schoeffel monochromator with a xenon arc lamp without extra interference filters.

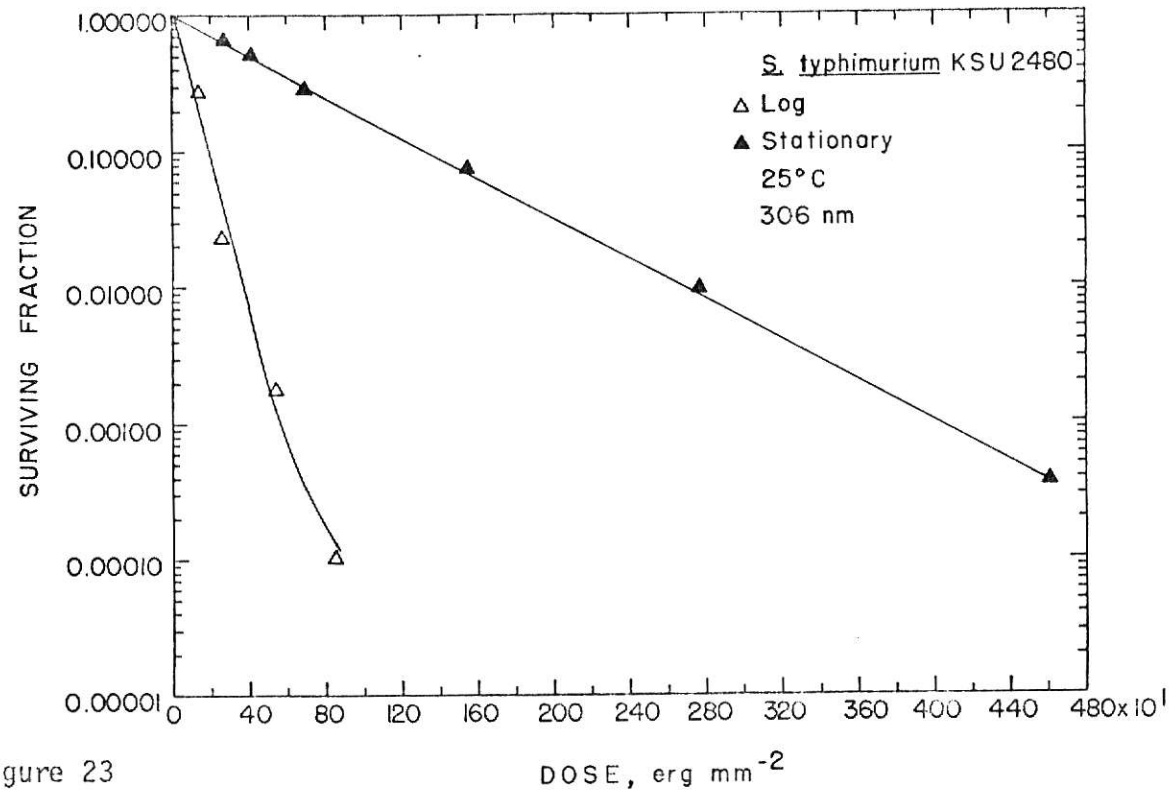


Figure 23

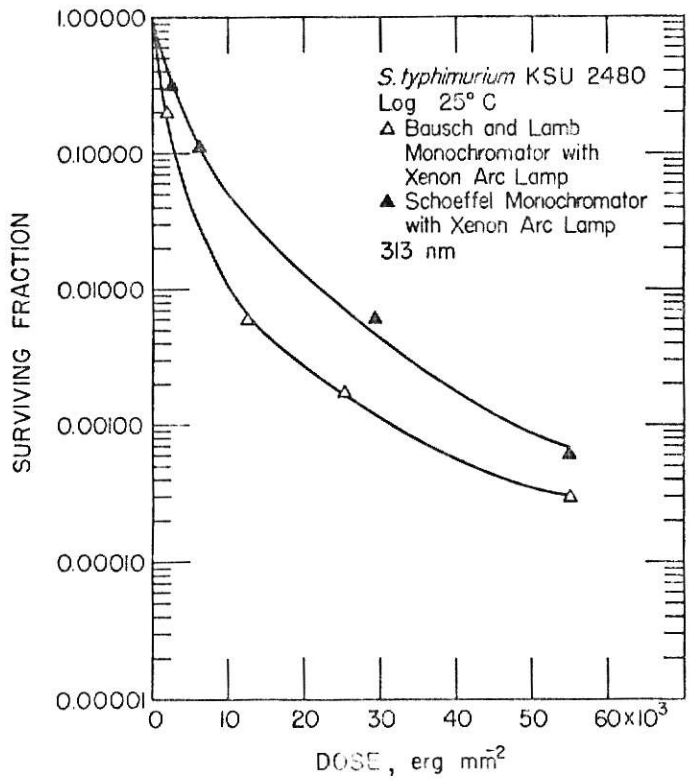


Figure 24

Figure 25

Inactivation curves of log phase cultures of Salmonella typhimurium KSU 2480 at 320 and 340 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

Figure 26

Inactivation curves of log phase cultures of Salmonella typhimurium KSU 2480 at 330 nm using a Bausch and Lomb monochromator with a xenon arc lamp and a Schoeffel monochromator with a xenon arc lamp without extra interference filters.

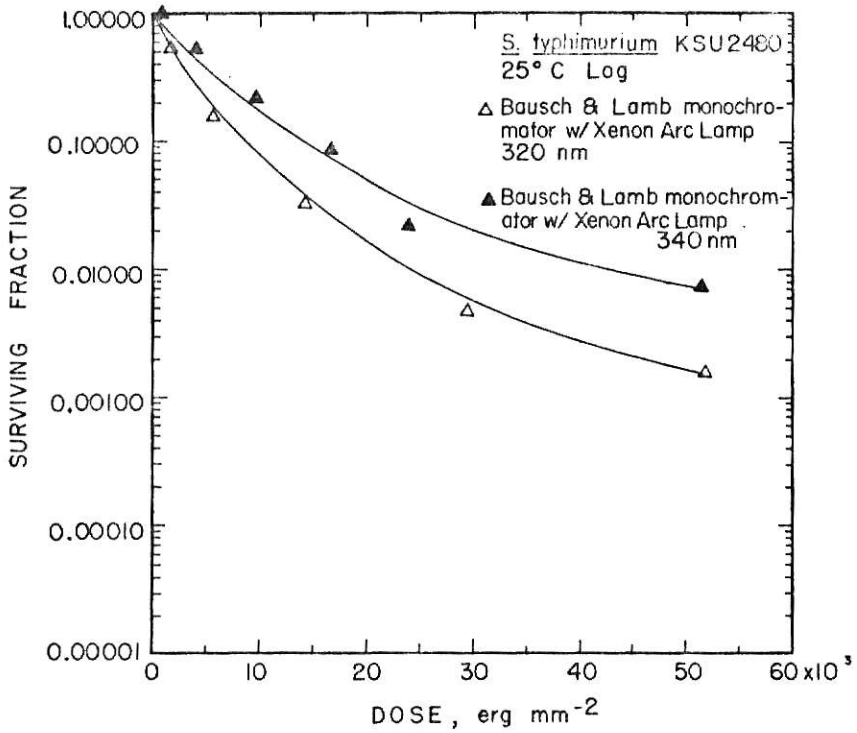


Figure 25

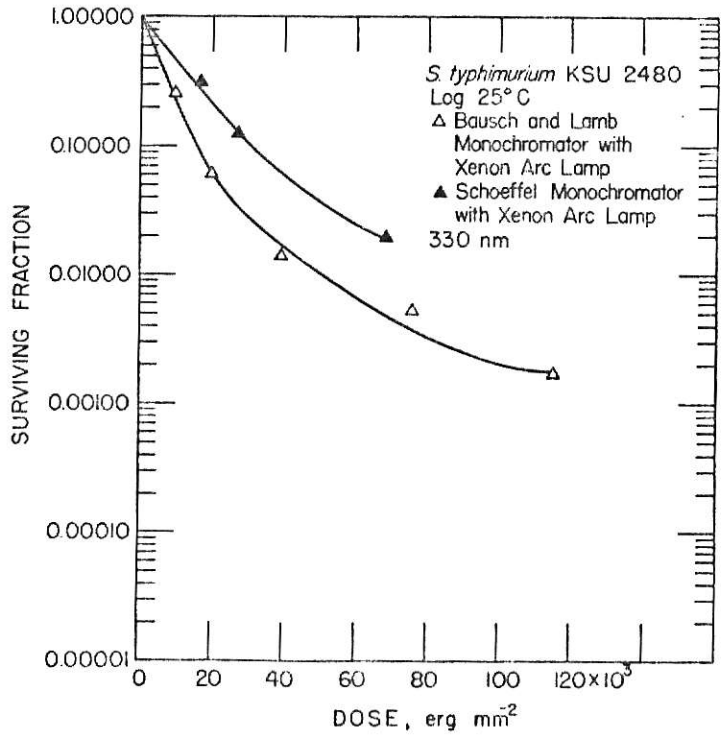


Figure 26

Figure 27

Inactivation curves of log phase cultures of Salmonella typhimurium KSU 2480 at 350 nm using a Bausch and Lomb monochromator with a xenon arc lamp and a Schoeffel monochromator with a xenon arc lamp without extra interference filters.

Figure 28

Inactivation curve of a log phase culture of Salmonella typhimurium KSU 2480 at 365 nm using a Bausch and Lomb monochromator with a xenon arc lamp without extra interference filters.

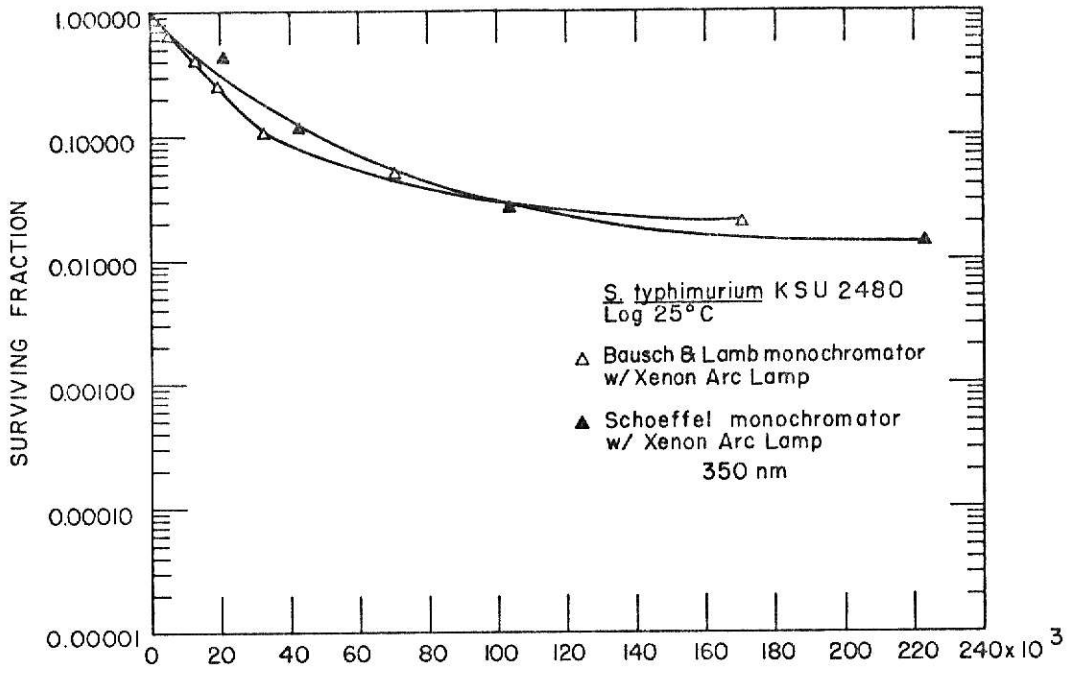


Figure 27

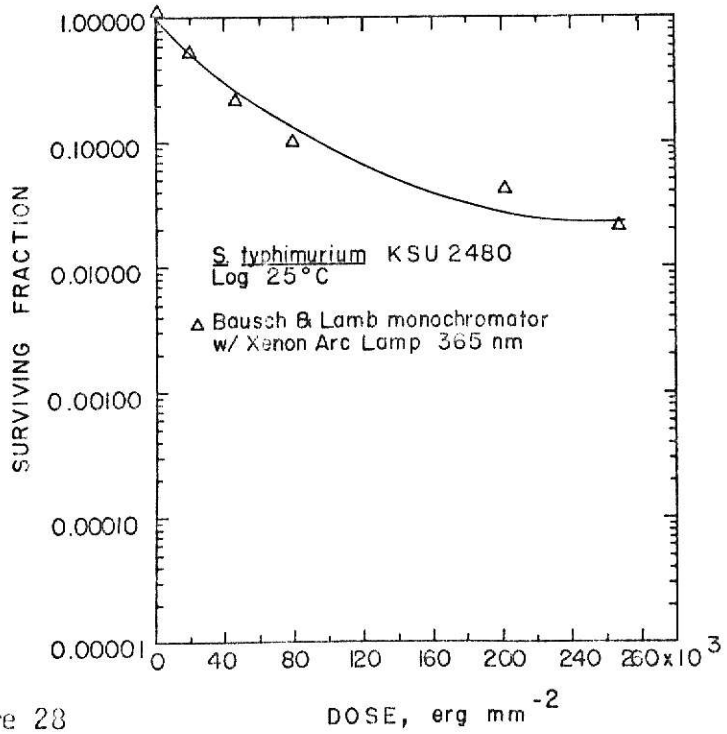


Figure 28

After examination of the curves it was decided to base the action spectra on the slope of exponential inactivation of the cells. For the purpose of standardization the slope at 10% survival was selected since not all curves are linear exponentials at survival fractions less than this. The value of the slope was calculated by $\frac{N}{N_o} = e^{-KD}$ where N_o is the number of active units initially present, N is the number remaining active after an incident dose D, and K is the slope and equals the inactivation cross-section. The value of the slope was then plotted versus wavelength to give the action spectra (Figures 29 and 30). The most efficient wavelengths for inactivation produce the greatest slopes.

Inactivation Using the Bausch and Lomb Monochromator with a Super Pressure Mercury Lamp

Exponential and stationary cultures of S. typhimurium were inactivated using a super pressure mercury arc lamp as the energy source at 313 and 365 nm. Interference filters were used to increase the monochromicity of the beam. Inactivation of E. coli was also obtained at 313 nm. These curves are shown in Figures 31, 32, and 33. It is important to note that at 365 nm the curve shape has changed from that obtained in previous experiments for the recA mutants. The slopes of the inactivation at 313 and 365 nm are plotted on the action spectra in Figures 29 and 30.

Inactivation Using the Schoeffel High Intensity Monochromator with a Xenon Arc Lamp

This monochromator was especially designed to give great amounts of energy and a very pure spectrum of light. Scattered light has been reduced to a minimum. Survival curves for log S. typhimurium using this light source and no extra filters at 313, 330, and 350 nm are shown in Figures 24, 26, and

27. Again the typical curve shape for the recA mutants is seen. These curves are in good agreement with the inactivation curves using the Bausch and Lomb monochromator with a xenon lamp. Cultures are only slightly more resistant using the Schoeffel monochromator as the light source when the slopes of inactivation are plotted on the action spectrum (Figure 29).

Inactivation studies of log and stationary cultures of S. typhimurium using the Schoeffel high intensity monochromator and interference filters or a cut-off filter 0140 are shown in Figures 34, 35, 36, and 37. At 330, 340, 350, and 365 nm the curve shape has changed. The slopes of the exponential inactivation of the cells are still plotted on the action spectra (Figure 29) but the curves are fitted to the following multi-target equation $S=1-(1-e^{-KD})^n$ (where S is the fraction surviving, D is the incident dose in erg/mm^2, K is the inactivation constant or slope in units of $(erg/mm^2)^{-1}$, and n is the shoulder constant) by the computer using a method developed by Tyler and Dipert (1962).

Inactivation Using the Marc 300 Lamp

Log and stationary cells of S. typhimurium were inactivated at 460 and 550 nm using the General Electric Marc 300 lamp and interference filters. The results are seen in Figures 38 and 30. The curve shape is consistent with those seen at 330, 340, 350, and 365 nm. Again the inactivation curves are fitted to the expression $S=1-(1-e^{-KD})^n$ to obtain the slopes of the exponential inactivation which are then plotted on the action spectra (Figure 29).

Effect of Temperature

To see if lowering the temperature had any effect on inactivation, log and stationary cells were inactivated at 254 and 365 nm under the conditions previously stated except the temperature was lowered to 0° C using a jacketed

irradiation vessel. Due to the fact that these inactivation curves are identical to those obtained at 25° C, the mechanism of inactivation is probably not enzymatic.

Division Delay

It is known that in the bacterium E. coli B there is a delay in the division of cells after exposure to 265 nm. After a dose of only 10 erg/mm^2 less than 10% of the cells are able to divide and most cells are observed to form long filaments as cytoplasmic synthesis continues in the absence of division. RecA mutants of S. typhimurium and E. coli were examined under a phase-contrast microscope after irradiation at 254, 365, and 290 nm. The phenomenon of filament formation was not observed, therefore irradiation does not cause a delay in the division of cells.

Figure 29

Action spectra for log and stationary phase cultures of Salmonella typhimurium KSU 2480. The slope of the inactivation is plotted versus wavelength.

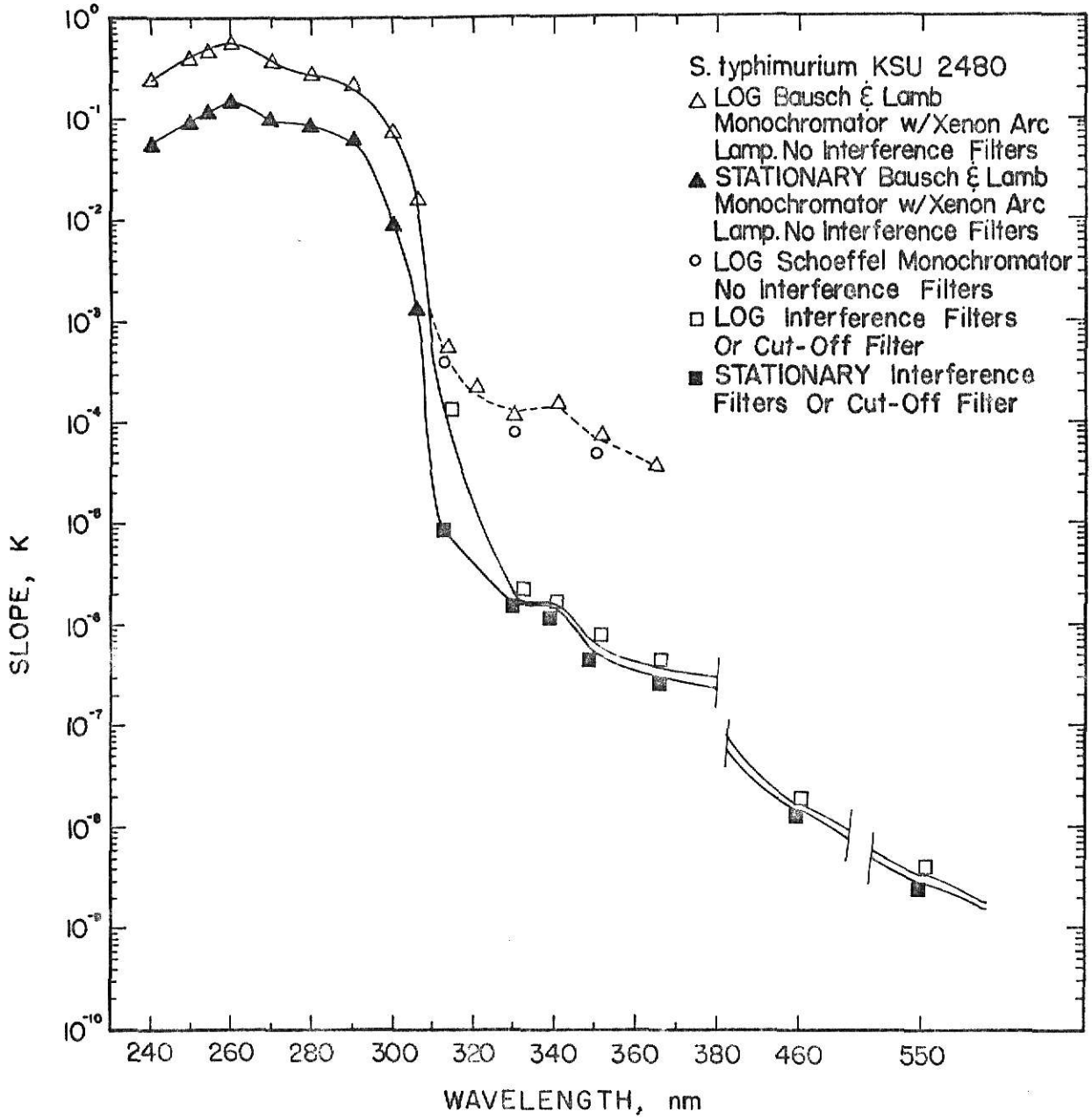

S. typhimurium KSU 2480
△ LOG Bausch & Lamb Monochromator w/Xenon Arc Lamp. No Interference Filters
▲ STATIONARY Bausch & Lamb Monochromator w/Xenon Arc Lamp. No Interference Filters
○ LOG Schoeffel Monochromator No Interference Filters
□ LOG Interference Filters Or Cut-Off Filter
■ STATIONARY Interference Filters Or Cut-Off Filter
SLOPE, K
WAVELENGTH, nm
240 260 280 300 320 340 360 380 460 550

Figure 29

Figure 30

Action spectra for log and stationary phase cultures of Escherichia coli K12 AB2463. The slope of the inactivation is plotted versus wavelength.

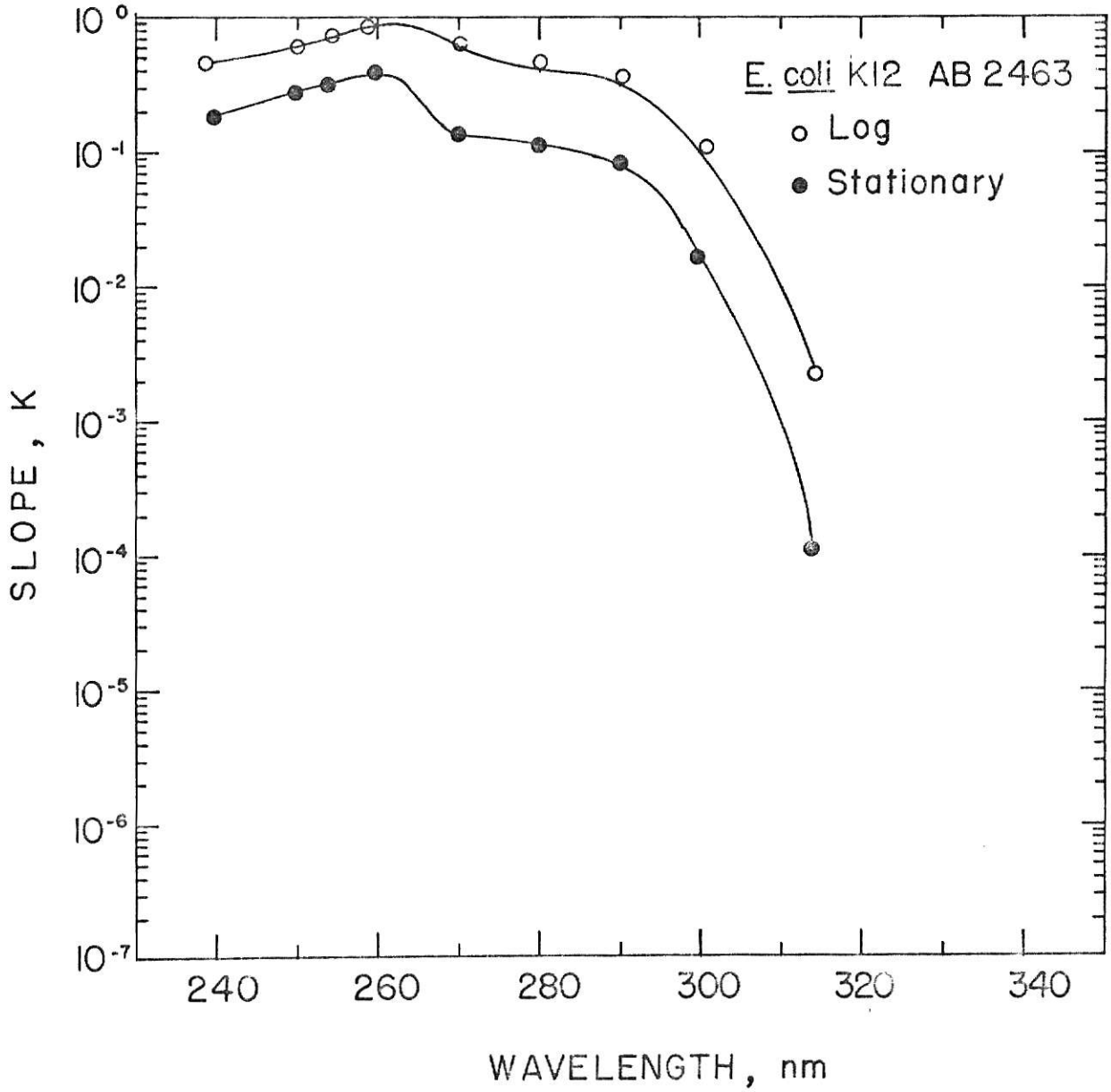

E. coli K12 AB 2463
Log
Stationary
SLOPE, K
WAVELENGTH, nm
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
240
260
280
300
320
340

Figure 30

Figure 31

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 313 nm using a Bausch and Lomb monochromator with a super pressure mercury lamp and the 313 interference filter. (Baird-Atomic).

Figure 32

Inactivation curves of stationary and log phase cultures of Escherichia coli K12 AB2463 at 313 nm using a Bausch and Lomb monochromator with a super pressure mercury lamp and the 313 interference filter (Baird-Atomic).

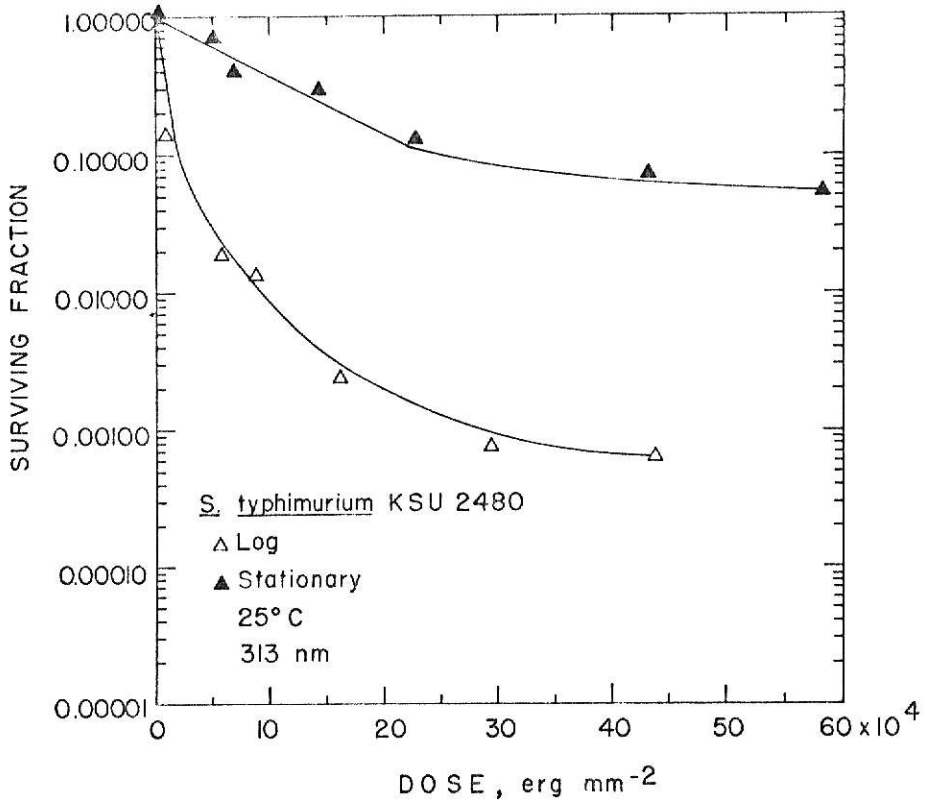


Figure 31

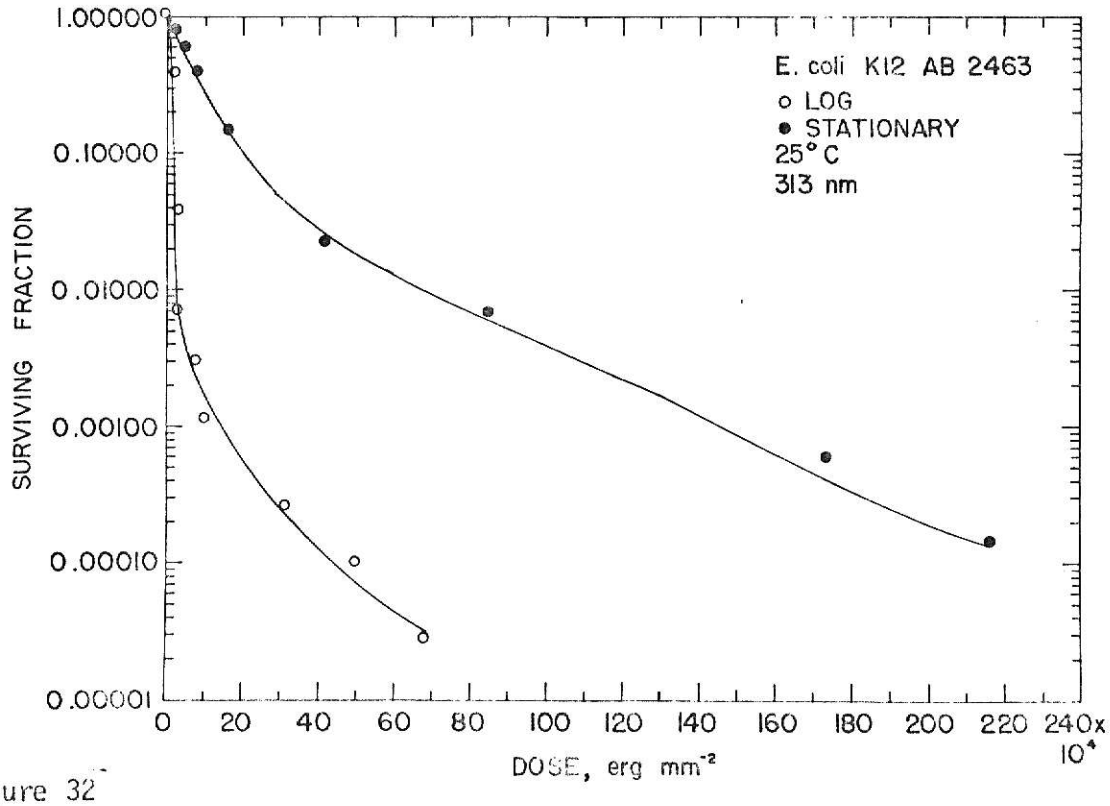


Figure 32

Figure 33

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 365 nm using a Bausch and Lomb monochromator with a super pressure mercury lamp and the 365 interference filter (Baird-Atomic).

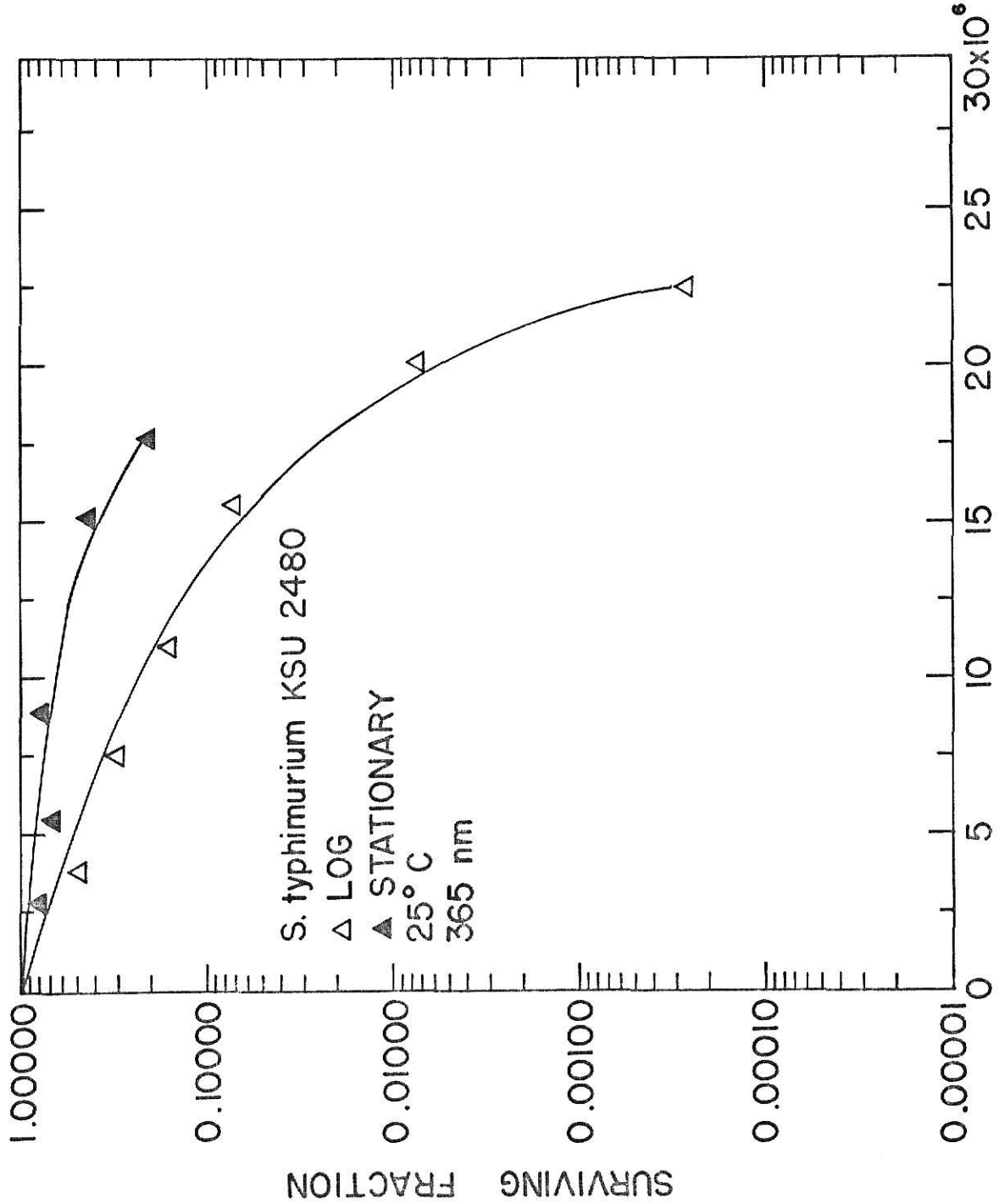

S. typhimurium KSU 2480
△ LOG
▲ STATIONARY
25° C
365 nm
SURVIVING FRACTION
1.00000
0.10000
0.01000
0.00100
0.00010
0.00001
0
5
10
15
20
25
30x10⁶
DOSE, erg mm⁻²

Figure 33

Figure 34

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 330 nm using a Schoeffel monochromator with a xenon arc lamp and the cut-off filter 0141 (Corning).

Figure 35

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 340 nm using a Schoeffel monochromator with a xenon arc lamp and the cut-off filter 0140 (Corning).

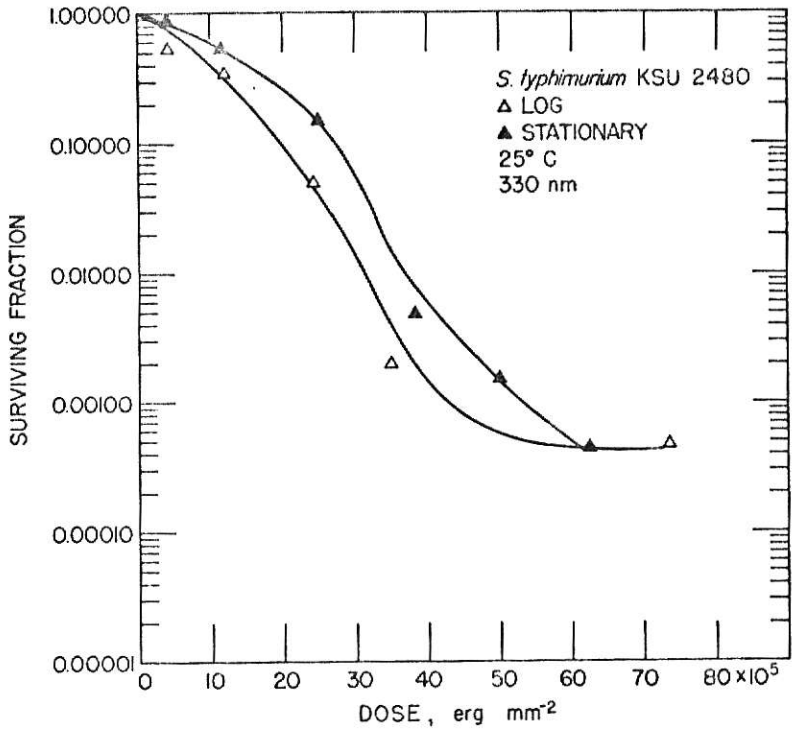


Figure 34

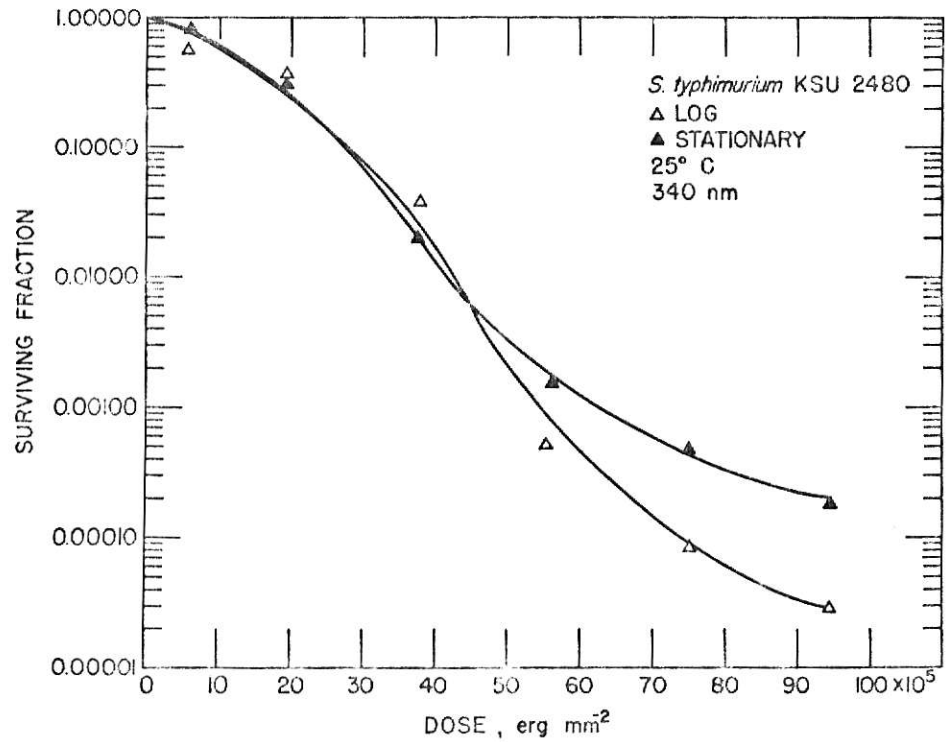


Figure 35

Figure 36

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 350 nm using a Schoeffel monochromator with a xenon arc lamp and the 350 interference filter (Baird-Atomic).

Figure 37

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 365 nm using a Schoeffel monochromator with a xenon arc lamp and the 365 interference filter (Baird-Atomic).

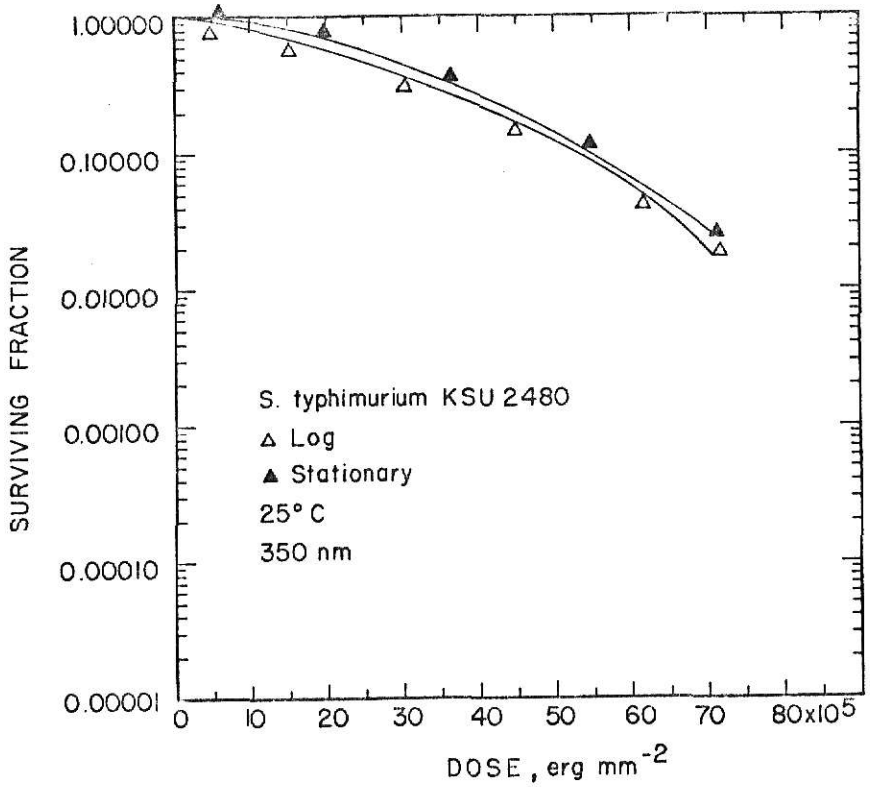


Figure 36

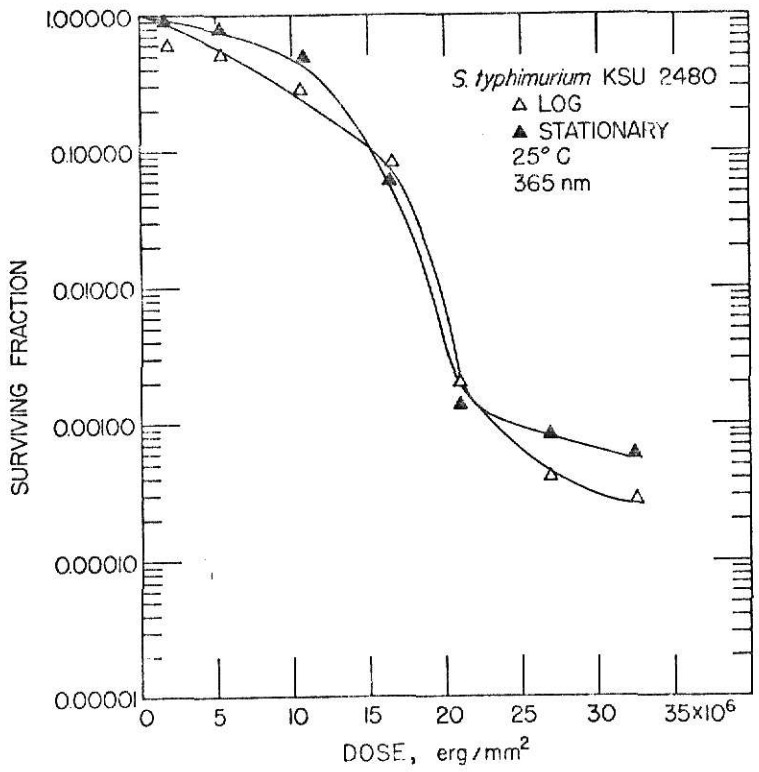


Figure 37

Figure 38

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 460 nm using the General Electric Marc 300 lamp and the 460 interference filter (Baird-Atomic).

Figure 39

Inactivation curves of stationary and log phase cultures of Salmonella typhimurium KSU 2480 at 550 nm using the General Electric Marc 330 lamp and the 550 interference filter (Baird-Atomic).

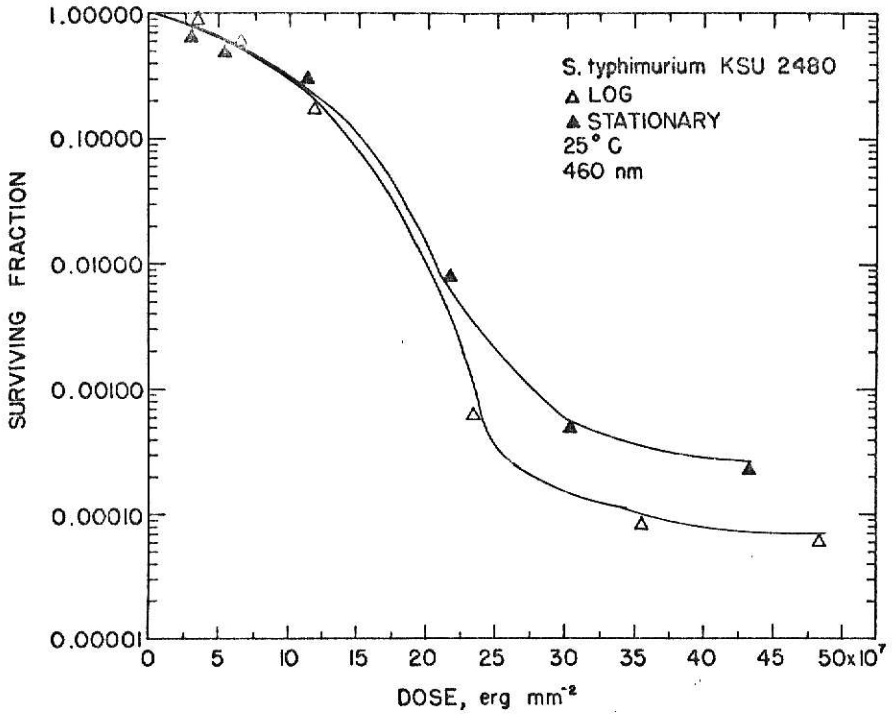


Figure 38

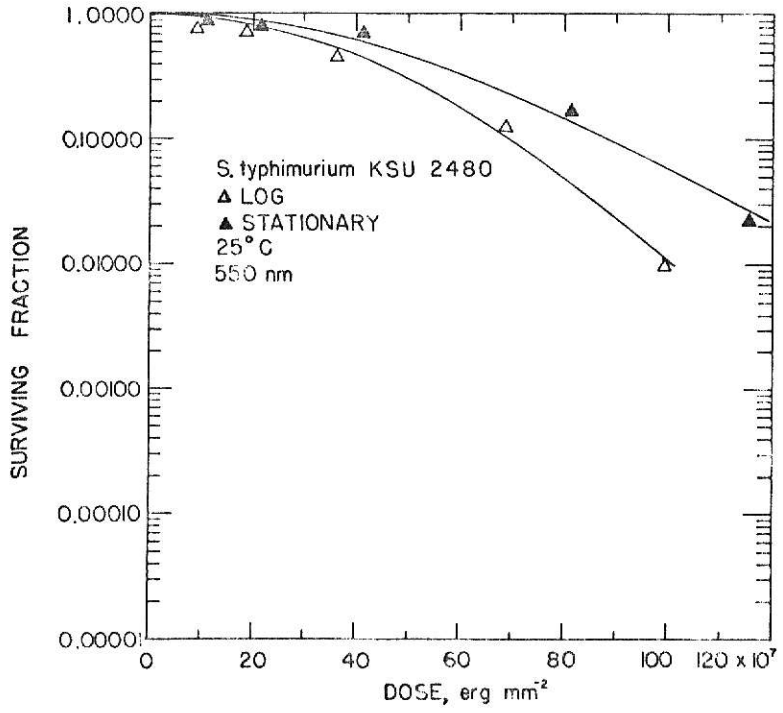


Figure 39

DISCUSSION

The characterization of the radiation absorbing chromophore(s) in the near-UV region was not obtained. However, as a result of these action spectra studies a more definitive description of the effect of monochromatic light in causing lethality in recombinationless mutants was obtained. Also, it has been shown that the great sensitivity of the recA mutant is not due to absorption of a single wavelength in the near-UV by the chromophore(s).

Action Spectra of RecA Mutants

Observation of the action spectra for inactivation by monochromatic light of both E. coli and S. typhimurium recA mutants are similar (Figures 29 and 30). This is not surprising since the chromosome-linkage maps of the two genera are similar (Sanderson, 1970; Taylor, 1970). Since these mutants have different nutritional requirements the assumption can be made that the mode of death is the same and is not simply due to toxic photoproducts in the media. That the action spectra of all recombinationless strains regardless of genus are the same is still open to question.

Evaluation of the Action Spectra in the Far-UV (240-300 nm)

In all four action spectra for log and stationary cells for both strains maximum inactivation using monochromatic sources occurs at 260 nm. Photochemical damage in the DNA is implicated as the primary target since the absorption spectrum of DNA also peaks at 254 nm. This correlates with all inactivation studies done with a variety of organisms. The types of specific chemical and physical changes in the DNA were not analyzed but thymine dimers are presumably the major photoproducts.

A very interesting observation and possibly a very important one for recA mutants is the shoulder seen at 270 to 290 nm which is not seen in action spectra of recA+ strains (Dr. R. B. Webb personal communication). This shoulder is consistent with a protein involvement in inactivation (Setlow and Boling, 1965). One possible explanation would be DNA-protein cross-linking. The chemical mechanisms by which DNA and protein are cross-linked is not yet known, however, the isolation of a mixed photoproduct of uracil and cysteine (5-S-cysteine, 6-hydrouracil) from the in vitro UV irradiation of a solution of uracil and cysteine may serve as a possible model for the cross-linking phenomenon (Smith and Aplin, 1966). A possible explanation may be that recA$^-$ strains are unable to dissociate DNA-protein cross-linking. Further experiments would include assaying for DNA cross-linked to protein at these wavelengths by the method described by Smith and Hanawalt (1969).

Log and stationary cultures (Figures 29 and 30) show similar sensitivities to inactivation in the far-UV with log cells only slightly more sensitive.

Evaluation of the Action Spectra in the Near-UV (300-380 nm)

It can be concluded from the action spectrum (Figure 29) that the sensitivity of the recA mutants varies under differing degrees of monochromicity in the 330-365 nm region. Log cultures (□) are one hundred times more resistant when inactivation experiments are done using interference filters which have a very narrow spectrum of light transmitted or the cut-off filter 0140. Although there is no conclusive proof, it is suggested that the great sensitivity of log recA mutants (Δ and ○) to near-UV depends on the absorption of more than a single wavelength of light. Without the interference filters a small amount of scattered light at all wavelengths is emitted from the monochromators. Log cultures (Δ and ○) are very sensitive under these conditions.

Inactivation by stray 254 nm is eliminated by the use of a pyrex cuvette. Examination of the transmission spectrum of the pyrex cuvette shows that wavelengths above 270 nm present in the scattered light would be transmitted through the cuvette. It is apparent from Figure 29 that inactivation using both the Schoeffel (○) and the Bausch and Lomb (△) monochromators without interference filters produce similar curves at 313 to 365 nm. This eliminates the possibility that the difference in sensitivity is due to difference in the instrumentation rather than the presence of scattered light.

It has been observed (Eisenstark, 1970) that log recA mutants are much more sensitive than stationary recA mutants when the polychromatic irradiation source is blacklight bulbs with a spectrum that peaks at 365 nm. The polychromatic source emits all wavelengths in the 300 to 440 nm region. In the presence of scattered lights the log recA mutants in these studies using monochromatic light are more sensitive than the stationary cultures in the 320-365 nm region. Stationary cultures are not inactivated under similar conditions which inactivate the log cultures. In the presence of interference filters which eliminate the scattered light, log (□) and stationary cultures (■) show similar sensitivity to monochromatic light in the 330 to 365 nm region. This provides further evidence for the previous postulation that the light sensitivity of recA mutants in the near-UV region is possibly due to multiple wavelengths since the great sensitivity of the log cultures is seen only when a small amount of scattered light is present. The greatest difference of sensitivity in log phase and stationary phase cultures in these studies using monochromatic light is seen at 306 nm at which their slopes of inactivation vary by a log and a half. It is of interest to mention that the

shoulder at 330 to 340 nm is seen in action spectra both with and without the presence of scattered light and is therefore real and not merely an artifact of the instrumentation. The significance of this shoulder remains to be investigated but it is thought that wavelengths in this region together with wavelengths in the 270 to 290 nm region might give increased inactivation. In addition no definite explanation can be offered at this time for the difference in the shape of the survival curves seen at 330, 340, 350, and 365 nm with interference filters or cut-off filter 0140. According to the classical target theory, a shoulder on a survival curve should indicate multiple targets (or multiple hits on targets). The shoulders have been reinterpreted as implicating the capacity of these cells to repair radiation damage (Haynes, 1964). The shoulder area of a survival curve thus may represent the dose range within which the cells can cope with the damage product.

At the time of this investigation interference filters were only available for 313, 350, 365, 460, and 550 nm. In the 240-310 nm region it is thought that inactivation with interference filters present would give action spectra similar to that obtained in Figure 29. This is justified by the fact that lethality of log recA at 313 nm in the presence (□) and absence (Δ and ○) of interference filters differ in sensitivity by only 2.5 fold. Had the slope of inactivation in the presence and absence of an interference filter at 313 nm differed by a log or more the justification could not have been made.

In conclusion it might be said that the slopes of inactivation obtained without interference filters in the 320-365 nm region are merely an artifact of the instrumentation. The true action spectra of lethality using monochromatic light would be that obtained using interference filters. The great sensitivity obtained without interference filters, however, demonstrates the possibility

of a multiple wavelength effect. It was hoped that there would be very sharp peak(s) in the action spectra with decisive inactivation, the fact that such peaks did not arise supports the view that inactivation may be a complex process.

Evaluation of the Action Spectra in the Visible (380-780 nm)

A plot of the slopes of the inactivation for S. typhimurium at 460 and 550 nm are seen in Figure 29. Log and stationary phase cultures exhibit similar sensitivity.

SUMMARY

Log and stationary phase cultures were inactivated by monochromatic light at selected wavelengths from 240 to 550 nm. The slopes of the exponential inactivation were plotted versus wavelength to give the action spectra. Log and stationary phase cells show similar sensitivity to light emitted at 240 to 290 nm in the far-UV, at 330 to 365 nm in the near-UV, and at 460 and 550 nm in the visible. The difference in sensitivity lies in the 300 to 313 nm region with log being more sensitive than the stationary.

As noted in previous studies the peak of inactivation is at 260 nm which corresponds to the absorption spectrum of DNA. A shoulder was observed in the 270 to 290 nm region which is not seen in action spectra of *recA*$^+$ strains. This shoulder possibly indicates DNA-protein involvement which may be DNA-protein cross-links. Another shoulder which cannot be explained was observed in the 330 to 340 nm region in the near-UV.

As a result of the studies presented in this thesis, it can be concluded that it is unlikely that the chromophore(s) absorbing the radiation in the near-UV which results in lethality will be identified by an action spectrum using monochromatic light.

ACKNOWLEDGEMENTS

The author wishes to express her sincere appreciation to her major professor Dr. A. Eisenstark for his guidance throughout this study.

Great appreciation is also expressed to Dr. R. B. Webb, Argonne National Laboratory, for encouragement, helpful discussions, and the use of his facilities.

Financial support was provided by a National Institutes of Health Traineeship in Genetics.

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ACTION SPECTRA OF LETHALITY IN A RECOMBINATIONLESS MUTANT OF *SALMONELLA TYPHIMURIUM*

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B. S., Kansas State University, 1970

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

ABSTRACT

The purpose of this investigation was to obtain an action spectrum of lethality for both log and stationary phase cultures of a recombinationless (recA) mutant of Salmonella typhimurium. Previous experiments have shown that recA mutants are highly sensitive to light emitted from blacklight and fluorescent bulbs (Eisenstark and Ruff, 1970). At the present time the chromophore(s) that absorbs the near-UV radiation or that passes the energy on to other molecules and thus causes lethality has not been identified. Action spectra of a relative biological or chemical response to different wavelengths have been useful in identifying chromophores when they correspond to absorption spectra of the chromophores.

Log and stationary phase cultures of a recA mutant of S. typhimurium were inactivated by monochromatic light at selected wavelengths between 240 and 550 nm. The inactivation curves up to 330 nm were not linear exponentials representing one hit-inactivation except for at least 10% survival in all curves. The typical curve shape has two parts: 1) an initial exponential; 2) a tailing effect with the slope decreasing at higher doses. It was proven that the curves did not represent a mixed population. The slope of the exponential inactivation at 10% survival of each curve was calculated by $\frac{N}{N_0} = e^{-KD}$ where N_0 is the number of active units initially present, N is the number remaining active after a dose D, and K is the slope. Survival curves at 330 nm and at higher wavelengths show a different curve shape. The slope of the exponential inactivation at these wavelengths were calculated from the multi-target equation $S = 1-(1-e^{-KD})^n$ where S is the fraction surviving, D is the incident dose in erg/mm^2, K is the inactivation constant or slope in $(erg/mm^2)^{-1}$, and n is the shoulder constant. The slope of the exponential

inactivation of all curves were then plotted versus wavelength to give the action spectra.

Maximum sensitivity was seen at 260 nm which corresponds in the shape of the curve and at its peak of sensitivity to the absorption spectrum of nucleic acids. A shoulder was observed in the 270 to 290 nm region which is possibly indicative of DNA-protein cross-linking. In the near-UV a shoulder was observed at 330 to 340 nm but no peak of maximum sensitivity was observed.

The results therefore indicate that the great sensitivity in recA mutants is not due to the absorption of a single wavelength by a single chromophore. The effect may be dependent on multiple wavelengths or multiple chromophores.