Bernard Adkins, Jr. CHARACTERIZATION OF THE PHOTOENZYMATIC REPAIR OF ULTRAVIOLET-INDUCED DAMAGE IN STAPHYLOCOCCUS AUREUS. (Under the direction of Wendall E. Allen) Department of Biology, March 1972.

The purpose of this study is to define any capacity of Staphylococcus aureus strain 7-8 to undergo photoenzymatic repair of UV-irradiation induced damage and compare it to the photoreactivation (PR) response of Escherichia coli strain B. Staphylococcus aureus showed greater inhibition by UV irradiation than E. coli. This was consistent with the higher adenine and thymine content of S. aureus. Staphylococcus aureus showed an enhanced rate of photoreactivation with no lag in initiation of the PR response with low PR doses compared to E. coli. Maximum PR capacity of both cultures was about equal and occurred for cultures incubated at 23-25 C. The PR responses at 11-12 and 35-37 C for S. aureus and E. coli differed, although both were capable of PR at all temperatures employed. The PR response of E. coli was directly related to the dosage of PR light (ergs/mm^2); however, the photoenzymatic capacity of S. aureus was not directly responsive to continued decrease in light intensity. The capacity of S. aureus to undergo liquid holding recovery (LHR) occurred within a narrow range (23-25 C), whereas E. coli underwent LHR at all but the highest temperatures (35-37 C). The LHR response of S. aureus was somewhat more effective than E. coli and did not show the direct response to increased liquid-holding period as E. coli.

The PR value of a white pigmentation mutant of S. aureus was different from the normally pigmented parent. The relation of pigment

production to photoenzymatic response of S. aureus requires further clarification.

The PR values were non-refractory to exposure of cells to PR conditions in the presence of antibiotics inhibiting protein synthesis. These preliminary results may indicate the constitutive occurrence of PR enzymes.

The differences observed in PR capacity and LHR response of E. coli and S. aureus to conditions of PR exposure and pigmentation were related to the natural habitats of these species.

CHARACTERIZATION OF THE PHOTOENZYMATIC REPAIR OF ULTRAVIOLET-INDUCED DAMAGE IN *STAPHYLOCOCCUS AUREUS*

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by

Bernard Adkins, Jr.

APPROVED BY:

SUPERVISOR OF THESIS Wendall E. Allen

Dr. Wendall E. Allen

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Graham J. Davis

Dr. Graham J. Davis

DEAN OF THE GRADUATE SCHOOL

John M. Howell

Dr. John M. Howell

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Dedicated

to

My Wife, Jeanne

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INTRODUCTION

The repair of ultraviolet (UV)-induced damage in deoxyribonucleic acid (DNA) by enzymatic photoreactivation has been extensively characterized in several genera of bacteria. Photoreactivation of UV-induced damage of the gram-negative, non-pathogenic bacterium, Escherichia coli was investigated by several workers. Photoreactivation in E. coli was shown to be dependent upon the photoreactivating light dosage and the temperature during photoreactivation, with results varying from culture to culture. Ikenaga et al. (1970) reported photoreactivation of the gram-positive, non-pathogenic bacterium, Staphylococcus epidermidis. There are no reports of the capacity of pathogenic Staphylococcus aureus to undergo photoreactivation of UV-induced damage.

This study was performed to define the parameters of the photoreactivation response of S. aureus to UV-induced damage and to compare any response of S. aureus to the established photoreactivation capacity of E. coli. The results of this study may have evolutionary implications. The capacity of S. aureus to repair UV-induced damage might be related to its internal parasitic existence.

LITERATURE REVIEW

Many of the biological effects, lethal (Gates, 1928) and mutagenic (Emmons and Hollaender, 1939), of ultraviolet (UV) radiation have been explained as resulting from nucleic acid absorption of photons (J. K. Setlow, 1966). There were distinct correlations between the survival of UV-irradiated cells and the production of certain types of photochemical damage in the deoxyribonucleic acid (DNA) of these cells. DNA did not exhibit the same sensitivity to UV irradiations under all experimental conditions and its sensitivity to photochemical changes were altered by a variety of techniques: biological-growth state of cells; chemical-base-analog substitutions; and physical-denaturation, freezing or drying (K. C. Smith and Hanawalt, 1969).

In addition to the sensitivity of DNA to UV irradiation, the ability of the cell to repair the damage must be considered. Most of the understanding today of the molecular bases of recovery mechanisms has come from studies of bacteria and their viruses. Hanawalt (1968) proposed three possible modes of molecular recovery:

(1) The damaged DNA may be restored to its functional state in situ. This may result from the activity of enzymatic mechanisms or by decay of the damage.

(2) Normal function may be restored by replacement of the damaged unit of the DNA (or system) with an undamaged unit.

(3) The damage may persist, but under certain conditions, function continues due to utilization of alternate systems.

Much of the following discussion will be concerned with the

microbial systems of radiation repair. Generalizations concerning the microbial systems may ultimately relate to more complex and highly evolved living systems (K. C. Smith and Hanawalt, 1969).

Ultraviolet-induced Physical and Chemical Changes in Nucleic Acids and Nucleic Acid Components

Effects of UV radiation on Ribose and Deoxyribose

Carbohydrates exhibited essentially no UV absorption above 230 nm and therefore showed no photochemical reactions when irradiated with light of wavelengths greater than 230 nm (K. C. Smith, 1964a). Prior reports of photochemical alterations of carbohydrates were questionable since filters were not used to eliminate wavelengths below 230 nm.

Shugar (1960) proposed indirect chemical changes in the carbohydrates of nucleic acids exposed to UV even though the absorption did not occur in the carbohydrates. An indirect effect of UV on deoxyribose was demonstrated in cells containing the base analog of thymine, 5-bromouracil (BU), in their DNA. BU was debrominated when exposed to UV and a uracil radical produced (Burr, 1968). A hydrogen atom was removed from the adjacent deoxyribose. This resulted in the destruction of the deoxyribose, the production of uracil, and eventually chain breakage in the DNA (K. C. Smith and Hanawalt, 1969).

Effect of UV radiation on Purines

The purines and all their derivatives were shown to be approximately ten times more resistant to photochemical change than pyrimidines (McLaren and Shugar, 1964; Wacker et al., 1964). Purines

exhibited photodynamic action, the photosensitized oxidation of a substrate (Burr, 1968). K. C. Smith and Hanawalt (1969) UV-irradiated poly dAT and reported energy transferred from adenine resulted in the production of thymine radicals.

Hydration Products of the Pyrimidines

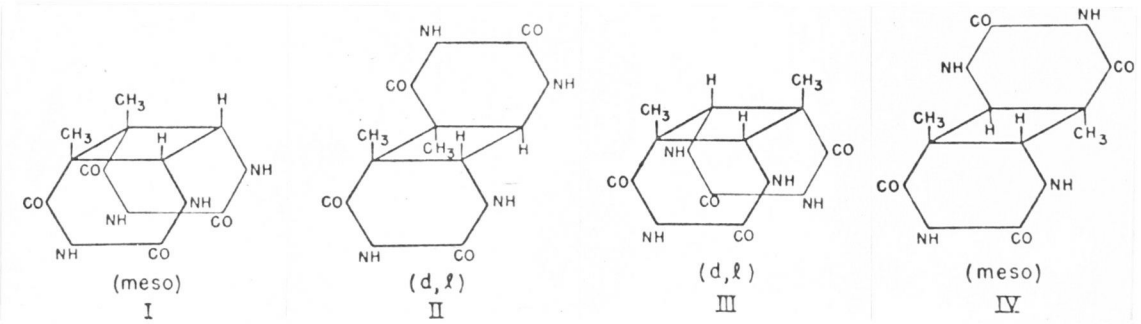
UV-irradiated solutions of uracil, cytosine and their derivatives lost their characteristic UV absorbance, but were reverted by heat, alkali, or acid treatment (Sinsheimer and Hastings, 1949). Hydration of the 5 - 6 double bond of uracil produced its changed absorption spectrum. Acid or heat dehydration reestablished the characteristic UV absorbance (Sinsheimer, 1954). Moore (1958) identified 6-hydroxy-5-hydrouracil as the reversible photoproduct of UV-irradiated uracil. Reversible photoproducts of other pyrimidine compounds were considered to be hydrates at the same position (Shugar, 1960). It was speculated that few if any pyrimidine hydrates were formed in irradiated native (double-stranded) DNA but were formed in single-stranded DNA (K. C. Smith, 1966).

Grossman's (1962, 1963) in vitro model system was used to demonstrate the possible importance of pyrimidine hydrates in causing mutations. The coding properties of UV-irradiated polycytidylic acid were changed: adenylic acid was substituted for guanylic acid in the purine polymer produced. This code change might have resulted from the formation of cytosine hydrates. Such mutations caused by hydrate formation may or may not be lethal (K. C. Smith and Hanawalt, 1969).

Thymine, Cytosine and Uracil Dimers

Pyrimidine dimers produced in UV-irradiated frozen thymine were

surveyed by J. K. Setlow (1966). Thymine dimers were isolated by ethanol extraction and recrystallized in water (Beukers and Berends, 1960). Infrared-spectrum analysis indicated the dimers contained a cyclobutane ring. Studies with nuclear magnetic resonance confirmed the cyclobutane structure (Wulff and Fraenkel, 1961) and the tentative structures of four cyclobutane-type thymine dimers are given below:



(from K. C. Smith, 1964a)

The cyclobutane-type thymine dimer was both formed and monomerized by UV radiation with differences in wavelength-dependencies for both the forward and reverse reactions (J. K. Setlow, 1966; R. B. Setlow, 1966). Long (280 nm) wavelengths produced more dimers than short (240 nm) UV wavelengths due to reversal of the reaction by irradiation at 240 nm. Dimerization was studied in: irradiated thymine (Wang, 1961; R. B. Setlow, 1961), thymine (K. C. Smith, 1964b; Weinblum and Johns, 1966), thymine dinucleotides (Johns, Rapaport and Delbruck, 1962; Sztumpf and Shugar, 1962; Deering and Setlow, 1963; Johns et al., 1964), polythymidylic acid (Deering and Setlow, 1963), and DNA (R. B. Setlow and Carrier, 1963; Wulff, 1963).

Four other types of pyrimidine dimers were found: uracil dimers (Wang, 1961; Smietanowska and Shugar, 1961), uracil-thymine dimers

(Beukers and Berends, 1960; Wacker et al., 1961), and cytosine-thymine dimers (R. B. Setlow, Carrier and Bollum, 1965). Uracil dimers were studied in UV-irradiated dUpU (deoxyuridylyl deoxyuridine, 3' → 5') by Helleiner, Pearson and Johns (1963) and in poly U by Grossman (1962) and Swenson and Setlow (1963). Cytosine dimers were difficult to detect because of spontaneous dehydration and deamination of cytosine when the 5 - 6 double bond was saturated (Green and Cohen, 1957). Irradiation of frozen cytosine (K. C. Smith, 1964a), CpC (cytidylyl-cytidine, Freeman, Hariharan and Johns, 1965), poly dI:dC (polydeoxyinosinic acid: polydeoxycytidylic acid, R. B. Setlow, Carrier and Bollum, 1965), or DNA (Dellweg and Wacker, 1962; Sauerbier, 1964; R. B. Setlow, Carrier and Bollum, 1965) produced cytosine dimers easily converted to uracil dimers by heat.

There were five types of pyrimidine dimers found in irradiated DNA: T$\hat{T}$, C$\hat{C}$, U$\hat{U}$, C$\hat{T}$, and U$\hat{T}$. All these dimers in the presence of the yeast enzyme were monomerized upon exposure to short (240 nm) UV wavelengths (J. K. Setlow, 1966).

Other Photochemical Reactions of Pyrimidines

Many pyrimidine photoproducts other than the hydrates or the cyclobutane-type dimers were produced *in vivo* and *in vitro* as a result or UV irradiation. These photoproducts appeared to be monomeric and dimeric in nature. They were recognized chromatographically, but their structures remain unknown. Literature concerning these photoproducts was reviewed by K. C. Smith and Hanawalt (1969). The biological importance of these photoproducts has not been determined to date.

Effect of UV radiation on the Molecular Weight of DNA - Chain Breakage

UV irradiation of nucleic acids caused a decrease in their viscosity and streaming birefringence probably resulting from depolymerization (Hollaender, Greenstein and Jenrette, 1941). This was confirmed by Moroson and Alexander (1961). Marmur and his co-workers (1961) found that more chain breaks were detected when DNA was denatured after irradiation. These results indicated most UV-induced breaks were in one chain of the DNA (J. K. Setlow, 1966).

Moroson and Alexander (1961) reported UV-induced chain breaks in DNA were oxygen-dependent. Marmur and his co-workers (1961) reported that UV inactivation of transforming DNA was oxygen-independent. The amount of oxygen required to produce a measurable number of chain breaks exceeded the range normally encountered in cells (Marmur et al., 1961; Freifelder and Davison, 1963). J. K. Setlow (1966) concluded chain breaks probably did not contribute to the biological effects of UV irradiation.

Other Effects of UV radiation on DNA

Other effects of UV radiation on DNA include: (1) DNA-DNA cross-links. UV-irradiation doses required to produce cross-links between strands were large (about 10^5 ergs/mm^2, Glisin and Doty, 1962). Bacteria were inactivated by 2 ergs/mm^2 (Hill and Simson, 1961; R. B. Setlow, Swenson and Carrier, 1963). Therefore, UV-irradiation induced DNA-DNA cross-links were of little biological significance except with highly UV-resistant transforming DNA (J. K. Setlow, 1966). (2) Cross-linking of DNA to protein. K. C. Smith and Aplin (1966) reported this cross-

linking phenomenon probably involved the formation of 5-S-cysteine-6-hydrouracil identified in an in vitro UV-irradiated mixture of uracil and cysteine. (3) The effect of base composition on the intrinsic sensitivity of DNA to UV irradiation. Sensitivity to killing by UV irradiation increased directly with the adenine-thymine content of the bacterial cultures studied (K. C. Smith and Hanawalt, 1969). (4) The effect of substitution by halogenated pyrimidines on the intrinsic sensitivity of DNA to UV irradiation. These pyrimidine analogs exhibited greater sensitivity to photochemical alteration than pyrimidines and seemed refractory to repair (K. C. Smith and Hanawalt, 1969). Szybalski (1962) described halogenated analogs of the natural deoxynucleotides, deoxyuridine (UdR) and thymidine (TdR): 5-fluorodeoxyuridine (FUdR), 5-chlorodeoxyuridine (CUdR), 5-bromodeoxyuridine (BUdR), 5-iododeoxyuridine (IUdR). The effect of presence of each of the above in DNA on its sensitivity to UV radiation was reviewed by K. C. Smith and Hanawalt (1969). (5) The influence of the environment during irradiation on the intrinsic sensitivity of DNA. The photochemical reactivity of thymine was markedly different when irradiated in solution, in frozen solution, or in dry films (K. C. Smith, 1964a). The photochemistry of DNA also varied with these environmental conditions during irradiation (K. C. Smith and Hanawalt, 1969).

Photochemistry of RNA

There is a paucity of literature on the photochemistry of RNA. Siegel and Wildman (1956) studied infectious RNA from Tobacco Mosaic Virus (TMV) and found RNA isolated from two different strains of TMV

differed more than five-fold in X-radiation sensitivity but were equally sensitive to UV radiation (also see McLaren and Takahashi, 1957).

Other work reported by K. C. Smith and Hanawalt (1969) on the photochemistry of RNA included: (1) The isolation of uracil dimers from irradiated RNA. (2) Photoreactivation (to be discussed later) of TMV-RNA. Hydrates rather than dimers may produce lesions responsible for inactivation of TMV-RNA. (3) Inactivation of transfer RNA from mouse liver. Photoproducts other than uracil dimers or hydrates formed during UV irradiation resulted in a changed secondary structure on the mouse-liver transfer RNA (K. C. Smith, 1964a). (4) The in vivo inactivation by UV radiation of the specific messenger RNA for an induced enzyme in Escherichia coli.

Recovery from Photochemical Damage: Repair Mechanisms

Recovery Due to Recombination

Two types of recovery due to recombination are multiplicity reactivation (MR) and cross reactivation. Multiplicity reactivation, first observed by Luria (1947), involved production of viable bacteriophage by host cells simultaneously infected with many UV-inactivated bacteriophages (Hanawalt, 1968). Many different bacteriophages and animal viruses demonstrated multiplicity reactivation (Drake, 1958). Multiplicity reactivation was observed in phage after inactivation by ^{32}P decay or after lethal treatments with x-rays, gamma rays, or nitrous acid (Hanawalt, 1968). The phenomenon probably involved random genetic recombination producing a functional genome from the undamaged components

of nonfunctional genomes (K. C. Smith and Hanawalt, 1969).

Cross reactivation or marker rescue also involved genetic recombination (Hanawalt, 1968). Multiple infection of a susceptible host with bacteriophages inactivated with high doses of UV radiation and normal non-irradiated phages resulted in recombination of genetic markers from the inactivated phage (Luria and Dulbecco, 1949; Doermann et al., 1955; Doermann, 1961).

Reversal of Damage by Repair in situ

Five types of reversal of damage by repair in situ have been identified: (1) decay of photoproducts by spontaneous reactivation, (2) thermal reactivation, (3) catalase reactivation, (4) direct photoreversal of pyrimidine dimers, and (5) enzyme-catalyzed photoreactivation. Decay of photoproducts involved their spontaneous reversion to the undamaged state (Hanawalt, 1968). Hydration products of pyrimidines were the only known photoproducts of DNA that decayed spontaneously (K. C. Smith, 1964a). Many different unstable photoproducts were formed within irradiated cells. Such photoproducts had to occur at a site of critical function to have a biological effect before decay (Hanawalt, 1968). K. C. Smith and Hanawalt (1969) therefore postulated actively growing cells (e.g. replicating DNA) less likely to benefit from spontaneous reversion than inactive cells.

Anderson (1949) demonstrated high temperatures produced increased decay rates for labile photoproducts. Thermal reactivation was studied in detail by Stein and Harm (1952). Hanawalt (1968) proposed elevated temperatures also enhanced the activity of the excision-repair system (to be discussed).

Catalase reactivation was observed by Monod et al. (1949). Ogg et al. (1956) found a mutant of E. coli unable to synthesize catalase due to a hemin deficiency, exhibited increased UV resistance when hemin was added to the growth medium. Latarjet and Caldas (1952) and Latarjet et al. (1954) proposed catalase action on organic peroxide-photoproducts accounted for reactivation. Rupert and Harm (1966) also suggested reactivation involved the reduction of UV-induced peroxides.

A fourth type of repair involved direct photoreversal of pyrimidine dimers. Thymine dimers were both produced and reverted to free thymines by UV absorption (Hanawalt, 1968). This equilibrium process was studied in detail by Johns et al. (1962). The absorption spectrum for dimers was approximately 1000 times greater at 239 nm than at 280 nm (R. B. Setlow, 1961). R. B. Setlow and Setlow (1962) indicated thymine dimers produced during UV irradiation of DNA at 280 nm caused over 50% loss of transforming capacity of bacterial DNA. Partial recovery of transforming capacity was obtained by re-irradiation at 239 nm. Prolonged irradiation at 239 nm eventually negated the reversal effect as measured by the transforming ability of DNA (R. B. Setlow and Carrier, 1963).

Although restoration of the transforming ability of DNA was used experimentally to study direct photoreversion, currently existing organisms appear unable to benefit from direct photoreversion. Direct photoreversion might have been important in the reversal of photo-damage in primitive organisms and could have been the precursor form of reactivation that evolved into the enzyme-catalyzed form of photoreactivation (K. C. Smith and Hanawalt, 1969).

The most thoroughly characterized cellular recovery mechanism from UV-radiation damage is enzyme-catalyzed photoreactivation. Photoreactivation was first observed by Whitaker (1941) in studies with Fucus eggs. The first individuals to rediscover and study the phenomenon were Kelner (1949) in bacteria and Dulbecco (1949) in bacteriophage. Illumination of irradiated cells with visible light activated enzymes involved in direct repair in situ of photoproducts produced by UV radiation in DNA (K. C. Smith and Hanawalt, 1969). Studies of enzyme-catalyzed photoreactivation involved (1) mode of enzyme action, (2) properties of the enzyme, and (3) general considerations about the in vivo process. The repair enzyme required 3 ev photons to undo damage in DNA produced by 5 ev photons (Hanawalt, 1968). Hidalgo-Salvatierra and McLaren (1969) reported the action spectrum for photoreactivation of UV-irradiated TMV-RNA did not resemble the action spectrum for photosynthesis by host cells. Isolated cellular extracts promoting photoreactivation in vitro were used extensively in characterizing photoreactivation (Goodgal et al., 1957; Rupert et al., 1958). Wulff and Rupert (1962) reported photoreactivating enzyme from yeast extract chemically converted thymine dimers directly to free thymine.

J. K. Setlow and Setlow (1963) reported transforming DNA exposed to 280 nm radiation and maximally subjected to enzymatic photoreactivation conditions was not further reactivated with 239 nm radiation. These results indicated reversal of dimers as the principle mechanism of enzymatic photoreactivation (K. C. Smith and Hanawalt, 1969). Transforming DNA irradiated at 239 nm was also enzymatically photoreactivated (J. K. Setlow and Setlow, 1963).

Extracts of both E. coli and baker's yeast exhibiting the capacity to photoreactivate damaged DNA in vitro were used extensively to characterize the photoreactivating enzyme. Rupert (1960) found the chemical specificities of the two systems apparently identical. Irradiated DNA maximally repaired by one system was not further repaired by the other. Baker's yeast extracts were preferred because extracts of E. coli required a dialyzable component in the reaction system (Hanawalt, 1968). Studies of photoreactivating enzymes reported in other organisms were reviewed by Rupert (1964).

Photoreactivating-enzyme specificity was extensively studied by Rupert (1961). He measured the extent to which various UV-irradiated substrates competitively inhibited enzymatic repair of UV-irradiated transforming DNA. Only polynucleotide strands containing adjacent pyrimidines showed photoreactivation. All combinations of pyrimidines formed dimers and were excised from DNA by enzymatic photoreactivation; however, thymine dimers were removed from DNA more efficiently than other dimers (J. K. Setlow et al., 1965). Only the types of DNA damage produced by UV radiation were enzymatically photoreversible (Hanawalt, 1968). The photoreactivating enzyme required a minimum of approximately nine nucleotides for recognizing and binding the UV-irradiated DNA (J. K. Setlow and Bollum, 1968). Harm et al. (1968) found approximately 20 photoreactivating enzyme molecules in stationary-phase cells of regulation defective mutants of E. coli B_{s-1}. Harm (1969) found an intense light flash of short duration (1 msec) resulted in repair of greater than 90% of enzyme-complexed photorepairable lesions between the temperatures of 37 C and -40 C in UV-irradiated transforming DNA of

Hemophilus influenzae. The action spectrum for enzymatic photoreactivation of Hemophilus influenzae transforming DNA by the E. coli extract had activity peaks at 360 and 390 nm (Rupert et al., 1958; J. K. Setlow, 1966). These peaks corresponded roughly to the absorption spectrum for enzymatic photoreactivation of E. coli B/r in vivo previously reported by Jagger and Latarjet (1956).

Harm and Hillebrandt (1962) isolated a mutant of E. coli B having no photoreactivation capacity. Extracts from this mutant lacked the ability to photoreactivate transforming DNA (Rupert, 1964; R. B. Setlow, 1964). Rupert and Harm (1966) reported this mutant did not repair photoreactivable damage in its DNA upon exposure to visible light after UV irradiation. However, Kaplan (1966) showed several UV-induced mutants of this mutant having no photoreactivation capacity were photoreversible. His studies suggested photoreactivation involved two enzymes (Kaplan, 1966). Support for this hypothesis was obtained by Ito et al. (1966). They reported photoreversible and nonphotoreversible mutagenic events induced by UV irradiation.

Reconstruction of Damaged DNA

Evidence for Excision-repair. R. B. Setlow et al. (1963) and Hanawalt (1968) showed UV-resistant E. coli B/r capable of removing or bypassing blocks imposed on normal replication by UV irradiation and resuming replication of DNA. Ultraviolet-sensitive E. coli B_{s-1} did not resume normal replication after UV irradiation. The number of UV-induced thymine dimers formed in each strain was approximately the same. Recovery of the capacity to replicate UV-irradiated DNA by UV-resistant E. coli B/r resulted from excision of thymine dimers from

damaged DNA during incubation in the dark (K. C. Smith and Hanawalt, 1969). Boyce and Howard-Flanders (1964) reported similar results for UV-radiation-resistant and senstivie strains of E. coli K-12. R. B. Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964) postulated defective regions in one of the two strands of DNA were excised and replaced with normal nucleotides using the complementary base-pairing information in the intact strand (Hanawalt, 1968). This mechanism was called "cut and patch" by K. C. Smith and Hanawalt (1969) and was used to explain the repair of a variety of structural defects in DNA. Pettejohn and Hanawalt (1964) obtained physical evidence for the repair replication or "patch" step.

Hanawalt (1968) used 5-bromouracil (BU) to study excision-repair replication of UV-damaged DNA. Newly replicated DNA was identified by placing cells in medium containing BU during and following UV irradiation. DNA sections containing BU were found. Excision-repair (dark repair) involved excision of UV-induced thymine dimers and repair replication of the sections of damaged DNA.

The Excision-repair Steps. (1) Recognition of the damaged region in the DNA. Many structural defects in DNA were recognized by the excision-repair system in addition to UV-induced pyrimidine dimers (K. C. Smith and Hanawalt, 1969). Repair replication was observed in bacteria following treatment with the following: nitrogen mustard, a bifunctional alkylating agent (Hanawalt and Haynes, 1965); nitroso-guanidine, a mutagenic agent (Cerda´-Olmedo and Hanawalt, 1967); and after thymine starvation in a thymine-deficient strain (Pauling and Hanawalt, 1965; Hanawalt, 1968). Other types of DNA damage recognized

and repaired included DNA degradation after mitomycin C treatment of bacteria (Boyce and Howard-Flanders, 1964), and DNA degradation after X-irradiation (Emmerson and Howard-Flanders, 1965; Hanawalt, 1968).

(2) Incision (enzymatic production of single-strand breaks near the damage in DNA). Following the recognition of damage in DNA, it is assumed that incision near the damage precedes the excision of the damaged region, but the two processes might normally occur as one single enzymatic process (Hanawalt, 1968). Moseley (1969) found an increased UV sensitivity in UV-sensitive mutants of Micrococcus radiodurans and speculated that this resulted from a deficiency in the incision step. Incision was demonstrated by Rörsch and his co-workers (1963, 1964, 1966) in a series of experiments with UV-irradiated double-stranded replicative form of bacteriophage ϕX174 (K. C. Smith and Hanawalt, 1969). Jansz and his co-workers (1963) showed this phage DNA was reactivated in spheroplasts of wild-type E. coli but not in mutants lacking the recognition step in the excision-repair process (Hanawalt, 1968). Incision occurred in DNA of UV-irradiated phage DNA upon exposure to cell free extracts but not in normal phage DNA (Rörsch et al., 1966).

The recognition step or incision step in excision-repair might not be required for the repair of damage resulting from treatment with ^{32}P, X-irradiation, or methylmenthanesulfonate, all of which cause single-strand breaks (Bridges and Munson, 1966; Searashi and Strauss, 1965; Hanawalt, 1968). Such deficient mutants could be UV sensitive and exhibit resistance to x-rays and methylmenthanesulfonate (K. C. Smith and Hanawalt, 1969).

(3) Excision and repair replication of damaged nucleotides. The processes of excision and repair replication of damaged nucleotides occur separately or simultaneously. K. C. Smith and Hanawalt (1969) indicated enzymes isolated from E. coli, exonuclease III and DNA polymerase, were ideal candidates for excision and repolymerization, respectively. Richardson and his co-workers (1964) proposed an in vitro model for the "cut and patch" process. Degradation of one strand of double-stranded DNA by exonuclease III resulted in loss of ability to transform cells. Transformation activity was restored by incubation with DNA polymerase (Hanawalt, 1968). Englund et al. (1969) showed DNA polymerase of E. coli had exonucleolytic activity in addition to polymerase activity. Peterson et al. (1971) found an exonuclease produced an extensive breakdown of UV- and X-irradiated DNA in the pol A1 mutant strain (deficient in DNA polymerase activity) of E. coli K-12.

Beattie and Setlow (1969) isolated an excisionless strain of Hemophilus influenzae. These cells were inactivated by exposure to UV-irradiated transforming DNA. This effect was not reversed by treatments of the transforming DNA known to produce maximum photoreactivation (Beattie and Setlow, 1969).

(4) Rejoining of the repaired segment to the continuous intact DNA strand. McGarth and Williams (1966) demonstrated rejoining of the repaired segment. An enzyme was isolated from extracts of E. coli capable of rejoining single-strand breaks in DNA (polynucleotide ligase activity; Gellert, 1967). The specificity of this joining enzyme indicated it was involved in the final step in DNA repair (Hanawalt, 1968).

Generality of Excision-repair. The excision-repair system was

observed in microorganisms other than E. coli. An extremely efficient excision mechanism was exhibited by the highly radioresistant bacterium, Micrococcus radiodurans (J. K. Setlow and Boling, 1965; Boling and Setlow, 1966). A strain of Mycoplasma laidlawii (PPLO) also exhibited excision-repair after UV irradiation (D. W. Smith, 1967; D. W. Smith and Hanawalt, 1967). Higgins and Sarachek (1970) reported temperature-dependent dark recovery (TR) from UV irradiation occurred widely among taxonomically unrelated yeasts. The process involved the correction or bypassing of a non-genetic form of inactivational damage. Repair replication was demonstrated in Tetrahymena pyriformis (protozoan) after UV treatment (Brunk and Hanawalt, 1967) and after X-irradiation (Hanawalt, 1968).

Garen and Zinder (1955) reported decreased survival of UV-irradiated Salmonella phage P22 in UV- or X-irradiated host cells. UV-irradiated phage survival increased upon infection of nonirradiated host cells. This phenomenon was called host cell reactivation (HCR) and was observed in other phages: λ, T1, T3, and T6 (Rupert and Harm, 1966).

Rasmussen and Painter (1966) demonstrated UV irradiation of HeLa cell tissue cultures stimulated a burst of DNA synthesis. Analysis of the replicated DNA by the density-labeling method indicated the DNA synthesis represented repair replication of the damaged DNA (Hanawalt, 1968). Regan et al. (1968) demonstrated removal of UV-induced thymine dimers from three human cell lines (RA, RAX10, and HeLa) in tissue culture.

Other Repair and Recovery Mechanisms

Liquid Holding Recovery. Liquid holding recovery (LHR) was

demonstrated in several microorganisms. Ultraviolet-irradiated E. coli B cells held in buffer (non-nutrient) for a time prior to plating exhibited a greater percentage of survivors compared to cells plated immediately after irradiation (Roberts and Aldous, 1949). The conditions for liquid holding recovery resulted in a delay in cell division following UV irradiation (Alper and Gillies, 1960). Jagger and his co-workers (1964) showed 4-6 hours liquid holding at room temperature allowed maximum recovery.

Ganesan and Smith (1968a, 1968b) isolated several recombination deficient (rec^-) mutants of E. coli K-12 that exhibited LHR. Genetic crosses indicated that LHR was due to the rec^- mutation or to some closely linked genetic marker (K. C. Smith and Hanawalt, 1969). K. C. Smith and Meun (1970) found the rec^- mutants of E. coli K-12 were more sensitive to UV irradiation than corresponding rec^+ derivatives. Such mutant strains would be expected to benefit from growth inhibition effects, if the rec^- strain deficiency involved the overtaxing of the excision-repair system (Hanawalt, 1968).

Indirect Photoreactivation. Jagger and Stafford (1965) observed indirect photoreactivation in mutants of E. coli B lacking the photoreactivating enzyme. These mutants exhibited a photoreactivation effect when irradiated with 334 nm light after UV irradiation (Hanawalt, 1968). This process was not temperature dependent, was not saturated with high intensities of radiation at 334 nm and, therefore, was probably non-enzymatic (K. C. Smith and Hanawalt, 1969). Jagger and Stafford (1965) also showed this process did not involve splitting thymine dimers. The 334 nm wavelength apparently inhibited growth

and division temporarily (Hanawalt, 1968). Patrick (1970) proposed that the cytosine-thymine precursor of the uracil-thymine adduct did not undergo enzymatic photoreactivation but was eliminated directly from UV-irradiated E. coli DNA by exposure to wavelengths around 313 nm. Jagger et al. (1969) proposed that indirect photoreactivation enhanced the effectiveness of the excision-repair system by delaying normal growth processes in UV-irradiated cells until repair was completed.

Photoprotection. Photoprotection (PP), discovered by Weatherwax (1956), was found similar to indirect photoreactivation in most respects (Hanawalt, 1968). Illumination of bacterial cells at 334 nm prior to UV-irradiation resulted in increased survival to UV radiation (Jagger and Stafford, 1962; Jagger, 1964). K. C. Smith and Hanawalt (1969) reported irradiation with 334 nm resulted in the irregular occurrence of PP in some bacteria. Hidalgo-Salvatierra and McLaren (1969) found that UV-inactivated TMV-RNA did not exhibit PP. Liquid holding recovery and PP exhibited similar effects (Jagger et al., 1964). Likewise, Castellani et al. (1964) reported LHR and photoreactivation in E. coli B exhibited similar effects. K. C. Smith and Hanawalt (1969) suggested pyrimidine dimers were being repaired by photoreactivation, LHR and PP. This indirectly implicated an excision-repair scheme operated in these processes and accounted for the growth inhibition effect.

Most of the knowledge of cellular repair processes to date resulted from studies of UV-sensitive mutants of gram-negative bacteria. The cellular repair processes of gram-positive bacteria have not been

extensively characterized. Jagger et al. (1970) found a 313 nm peak in the action spectra for photoreactivation of Streptomyces griseus and Micrococcus luteus. Similarly, Ikenaga et al. (1970) found a 313 nm peak in the action spectrum for photoreactivation of Staphylococcus epidermidis. Pillich et al. (1969) found Staphylococcus aureus exhibited HCR of UV-inactivated Staphylococcus phages A/5 and ϕ131. Characterization of DNA dark-repair processes in S. aureus was reported by Goering and Pattee (1971). Photoenzymatic repair of UV-irradiation damage of S. aureus was not reported to date.

MATERIALS AND METHODS

Cultures

The culture of Staphylococcus aureus strain 7-8 used throughout this investigation was isolated from the infected teat of a cow with mastitis. The culture of Escherichia coli strain B (ATCC 11303) was purchased from American Type Culture Collection, Rockville, Maryland. The cultures were maintained at 4 C on Trypticase Soy Agar, TSA, Baltimore Biological Laboratories (BBL), Baltimore, Maryland, containing 0.5% Yeast Extract (Difco).

Ultraviolet-irradiation Technique

The cultures of S. aureus and E. coli were inoculated separately into 10 ml of BBL Trypticase Soy Borth (TSB) containing 0.5% Yeast Extract and incubated at 37 C for 18-24 hours. The cells were collected by centrifugation (International Clinical Centrifuge, Model CL) at 1500 × g for 10 minutes. The cells were washed twice with 10-ml portions of sterile phosphate buffer (0.01 M, containing 100.0 ml of 0.01 M KH_2PO_4 and 63.1 ml of 0.01 M K_2HPO_4, pH 7.0) to remove nutrients and resuspended in 10 ml of the same buffer. Samples from each cell suspension were removed prior to all irradiations and the viable cell titers per ml were determined. The samples were diluted with 0.85% sterile saline to yield approximately 30-300 bacteria/ml and pour plates were prepared by adding approximately 20 ml of melted and cooled TSA fortified with 0.5% Yeast Extract to 1-ml amounts of appropriate dilutions in sterile petri plates (100 × 15 mm). All plates, regard-

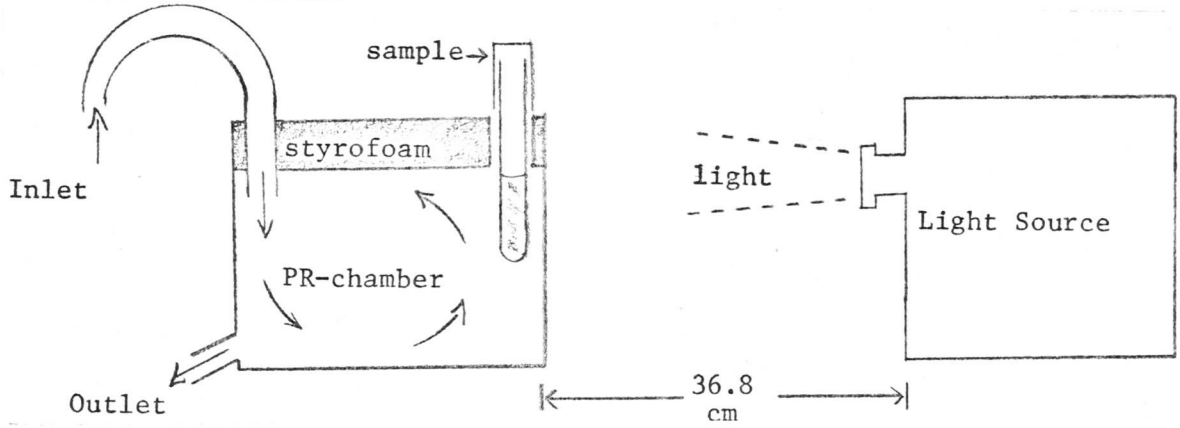
less of treatment of the sample contained, were incubated in the dark at 37 C for 48 hours. Colonies that developed on the plates were counted with an electric digital counter (American Optical Company, Model QR-1). This technique was used to determine the viable cell titers for all experiments. These buffer suspensions, containing approximately 10^9 bacteria/ml, were equally divided into two aliquots prior to exposure to UV irradiation. All irradiation procedures and subsequent incubations were performed in the dark. Each aliquot was transferred to a sterile, plastic (100 × 15 mm) petri plate (Kimble Products, Toledo, Ohio). The cell suspension in one plate was irradiated at 254 nm (Sylvania Electric Products, Inc., germicidal lamp, Model G815, 8 Watts) at room temperature (22-25 C) on an electric rotary shaker (1 cycle/second) 12 cm below the UV lamp. The other aliquot was treated in the same manner but was not irradiated. After treatment, viable cell titers of each aliquot were determined.

The lethality of the UV-irradiation dose employed was determined by measuring the viable cell titer at 10-second intervals during irradiation.

Photoreactivation Technique

Immediately after irradiation at 254 nm, the cell suspensions were divided equally into sterile test tubes (Pyrex). These tubes were placed in the constant-temperature chamber for photoreactivation (PR). This chamber consisted of a square, Pyrex chromatographic jar (3 gallon, Fisher Scientific Company) enclosed on three sides by light-reflective aluminum foil. The top made of styrofoam (1 inch thick)

served as the tube holder. The desired temperature was maintained by adjusting the ratio of hot to cold water flowing into the chamber from a common nozzle.



Photoreactivation Apparatus

The cells to be photoreactivated were immediately exposed to white light from a quartz-iodide lamp (Sylvania Electric Products, Inc., Type BCK, 500 Watts) housed in a Bausch and Lomb Slide Projector (Balomatic, Model 655). The wavelength range emitted by this lamp was from high UV to infrared wavelengths (approximately 300-750 nm). The Pyrex glassware used throughout the process absorbed all wavelengths below approximately 275 nm (Smith and Hanawalt, 1969). The time required for PR was varied experimentally. All extraneous light was excluded from the laboratory by covering all windows with light-impermeable aluminum foil and sealing door closures with vinyl-blub weather stripping.

Nonphotoreactivated (NPR) samples were covered with light-impermeable aluminum foil and treated exactly as the photoreactivated sample. After the specified time of exposure to light had elapsed, viable cell titers were determined of photoreactivated and NPR suspensions.

Liquid Holding Recovery (LHR)

A sample of the cells was removed after irradiation and transferred to a sterile test tube, covered with light-impermeable aluminum foil and placed in the PR chamber. The time this sample was held before plating was the same as the PR-exposure period. Capacity of the cells to undergo LHR was determined at three temperature ranges: 11-12 C, 23-25 C, and 35-37 C. These LHR samples served as NPR controls for the three temperatures indicated. Samples exposed to these temperatures were removed after a specified time and viable cell titers determined.

Effect of Exposure Time to White Light on PR

The exposure time to light used for PR varied from 5 to 180 minutes for both E. coli and S. aureus. To observe the effects of dosage of light on PR, viable cell titers of cultures exposed and not exposed to light were determined after the various time periods.

Effect of Temperature on PR

The percent PR obtained at three temperature ranges (11-12 C, 23-25 C, and 35-37 C) was determined. Temperatures were maintained in the PR chamber by temperature-controlled running water or the addition of ice for those below ambient. Viable cell titers were determined after PR at the various temperatures.

Effect of Light Intensity on PR

Light intensity was controlled by using glazed glass filters (white light) and a blue filter (peak transmittance at 450 nm, CBS Blue-450, obtained from Carolina Biological Supply Company, Burlington, North Carolina). Samples were exposed for 60 minutes to the following light intensities: white light, full intensity; white light, 50%

full intensity; white light, 1% full intensity; blue light, filtered-full intensity. Controls consisted of samples not exposed to light (NPR). Viable cell titers of photoreactivated and NPR samples were determined.

Effect of Pigmentation in Staphylococcus aureus on PR

A phosphate buffer suspension of the pigmented culture of S. aureus was irradiated with UV light (254 nm) using the previously described procedure. The irradiated samples were diluted and pour plates were prepared as before. White chromogenic mutant colonies that developed on the plates were isolated by streaking onto medium (TSA fortified with 0.5% Yeast Extract) and incubated at 37 C for 48 hours. Stable white mutants were inoculated into 10 ml of TSB containing 0.5% Yeast Extract and incubated at 37 C for 48 hours. A comparison was made of the percent PR of golden pigmented and white chromogenic mutants of S. aureus with E. coli.

Effect of Enzyme Synthesis on PR

Two antibiotics known to inhibit protein synthesis were used for this investigation: D (-) threo-2,2-dichloro-N-[β-Hydroxy-α (hydroxymethyl)-p-nitrophenethyl]-acetamide (Chloramphenicol, Sigma Chemical Company, St. Louis, Missouri) and 3'-(α-amino-p-methoxyhydrocinnamamido)-3'-deoxy-N,N-dimethyladenosine (Puromycin, Nutritional Biochemical Company, Cleveland, Ohio). Increasing concentrations of each antibiotic were prepared and added to tubes containing 10 ml of TSB with 0.5% Yeast Extract and inoculated with 0.1 ml of 18-hour TSB culture of E. coli and S. aureus. Minimal concentrations of the

antibiotics which inhibited growth were determined after incubation for 48 hours at 37 C.

Minimal inhibitory concentrations of the antibiotics were added to samples of the non-irradiated cells and the photoreactivated and NPR cells immediately after UV irradiation. Immediately following the PR period, all cell suspensions were centrifuged (1500 × g for 10 minutes) and resuspended to original volume in sterile phosphate buffer (0.01 M, pH 7.0). The viable cell titer of each cell suspension was then determined.

Calculations

All light intensity measurements (UV, white light and blue light) were determined with a Radiometer (Model 65, Yellow Springs Instrument Company) in ergs/cm^2/sec. These values were converted to ergs/mm^2/sec by dividing by 100.

The percent PR (%PR) was determined as follows:

$$\%PR = \frac{A - B}{C - B} \times 100$$

A is the average viable cell count from cell suspensions irradiated with UV light and subsequently exposed to white or blue light for the specific time periods. B is the average viable cell count from cell suspensions irradiated with UV light and not exposed to white or blue light, but held in the dark for the same time period as A. C is the average viable cell count from cell suspensions not irradiated with UV light and not exposed to white or blue light, but held in the dark for the same time period as A.

The percent LHR (%LHR) was determined as follows:

$$\%LHR = \frac{B - D}{B} \times 100$$

D is the average viable cell count from suspensions immediately after UV irradiation.

The percentage of the cells exposed to UV irradiation that survived (E) was determined as follows:

$$E = \frac{B}{C} \times 100$$

All %PR values were normalized to 0.1% survival as follows:

$$\frac{E}{\%PR} = \frac{0.1\%}{X}$$

$$X = \frac{(0.1\%)(\%PR)}{E} = \text{PR value normalized to 0.1\% survival.}$$

All %LHR values were normalized to 0.1% survival as above by substituting %LHR values into the equation for %PR values.

RESULTS

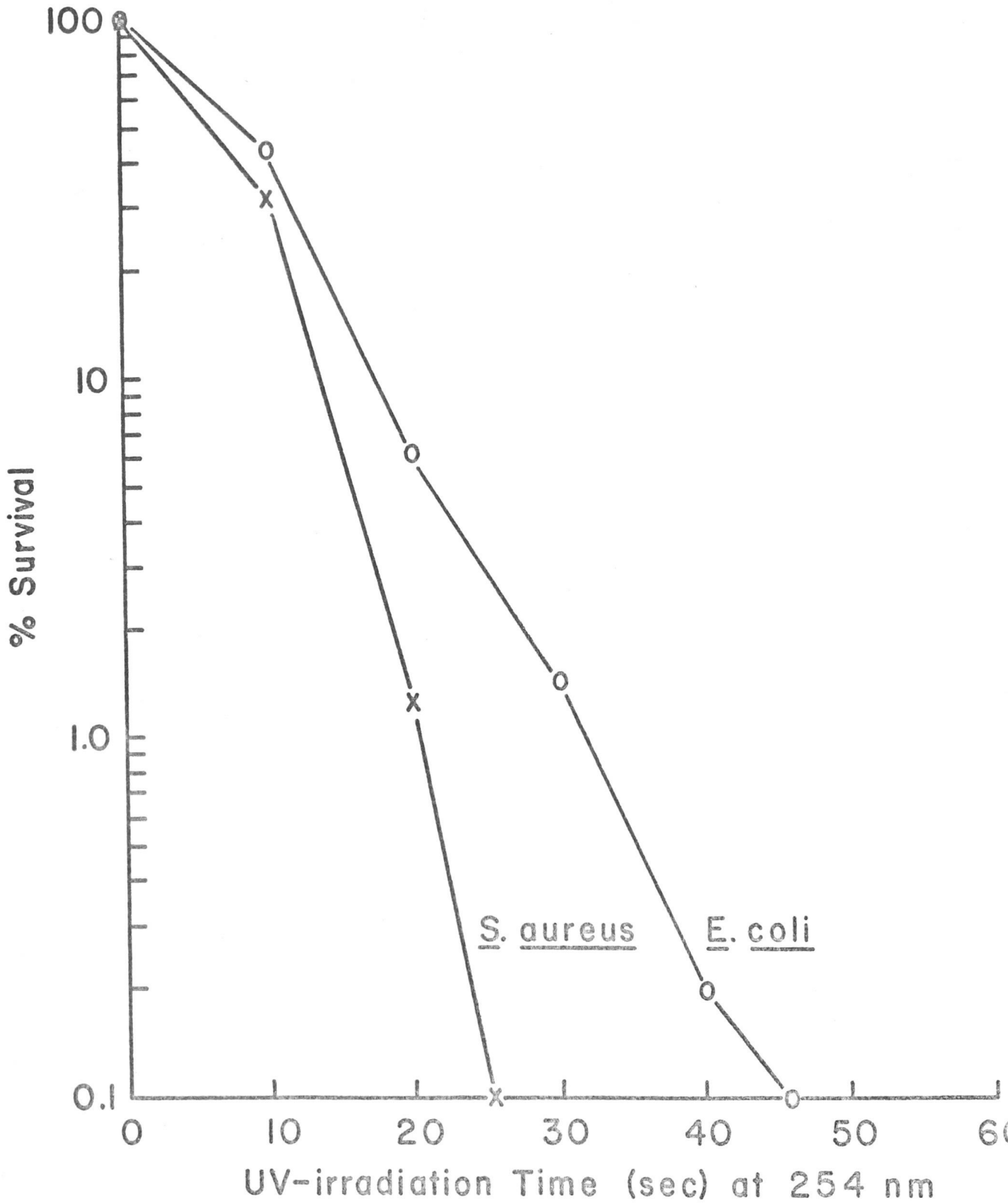
Figure 1 shows the effect of UV-irradiation (254 nm) time (seconds) on the percent survival of Escherichia coli strain B and Staphylococcus aureus strain 7-8. The results indicated UV-irradiation dosages of 3.15×10^3 ergs/mm^2 (45 seconds) for E. coli and 1.75×10^3 ergs/mm^2 (25 seconds) for S. aureus were required for 99.9% lethality.

The PR dosages used in all PR experiments with E. coli and S. aureus are given in Table 1. The dosages are given for all PR periods used. The dosages varied with intensity and refer to white light unless indicated otherwise.

The results of nine experiments on the effect of increasing PR dosage at 23-25 C on PR of E. coli and S. aureus are given in Figure 2. All PR values represent average values and were normalized to 0.1% survival. S. aureus exhibited a greater rate of PR than E. coli. E. coli showed an initial drop in response to PR compared to S. aureus. The PR values for both cultures increased rapidly during the first 90 minute exposure period. Both samples exhibited only gradual increases in PR after 90 minutes. Maximum PR percentages for both cultures were not significantly different. Experiments were terminated after 120 minutes with S. aureus and 180 minutes with E. coli.

The results of liquid holding at three temperatures on the recovery of E. coli and S. aureus from UV-irradiation damage are shown in Table 2. All LHR values were normalized to 0.1% survival. E. coli exhibited 0.01% LHR and S. aureus exhibited 0.05% LHR after 15 minutes at 23-25 C. E. coli exhibited 0.09% LHR and S. aureus exhibited 0.05%

Figure 1. Survival of Escherichia coli and Staphylococcus aureus to UV irradiation (254 nm) at 24 C.


100
10
1.0
0.1
% Survival
0
10
20
30
40
50
60
UV-irradiation Time (sec) at 254 nm
S. aureus
E. coli

LHR after 30 minutes at 23-25 C. *E. coli* exhibited 0.13% LHR and *S. aureus* exhibited 0.08% LHR after 60 minutes at 23-25 C. Neither culture exhibited LHR at 35-37 C after 15 minutes. *E. coli* exhibited 0.11% LHR at 11-12 C after 15 minutes while *S. aureus* exhibited no LHR at this temperature.

Table 1. Photoreactivation dosages.

Intensity and Color	PR Period (minutes)	Dosage (ergs/mm^2)
Full, White light	5	2.76×10^5
	10	5.52×10^5
	15	8.28×10^5
	20	1.10×10^6
	30	1.66×10^6
	40	2.21×10^6
	50	2.76×10^6
	60	3.31×10^6
	90	4.97×10^6
	120	6.62×10^6
	150	8.28×10^6
	180	9.94×10^6
50% Full, White light	60	1.62×10^6
1% Full, White light	60	3.60×10^4
Filtered-full intensity, Blue light	60	3.60×10^4

Figure 2. Effect of length of exposure to white light at 23-25 C on the PR of Escherichia coli and Staphylococcus aureus.

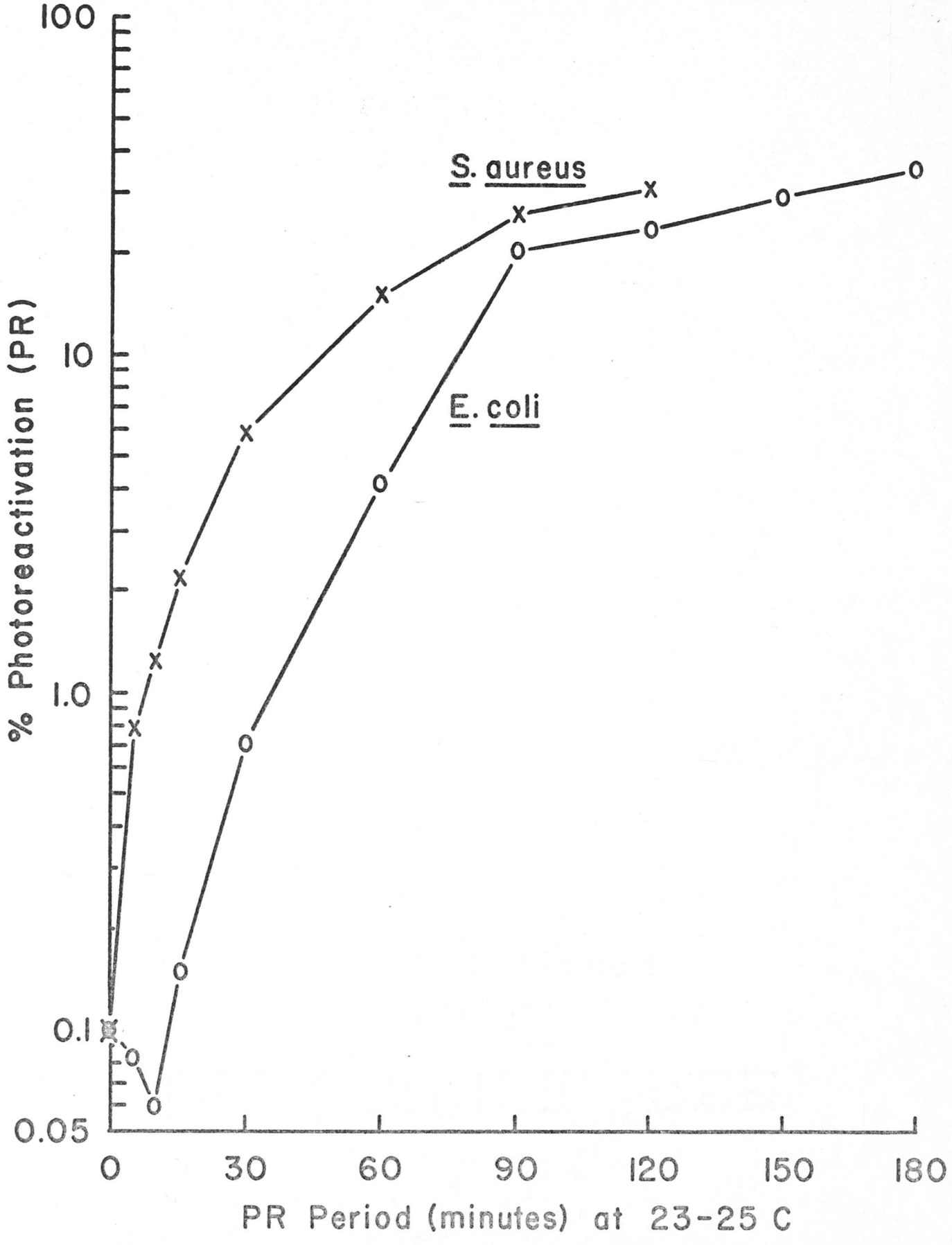

100
10
1.0
0.1
0.05
% Photoreactivation (PR)
S. aureus
E. coli
0
30
60
90
120
150
180
PR Period (minutes) at 23-25 C

Table 2. Liquid Holding Recovery (LHR) of Escherichia coli and Staphylococcus aureus.

Temperature Range (C)	LHR Period (minutes)	% Survivors	% LHR	
			E. coli	S. aureus
11-12	15	0.1	0.11	0
23-25	15	0.1	0.01	0.05
	30	0.1	0.09	0.05
	60	0.1	0.13	0.08
35-37	15	0.1	0	0

The effects of temperature on PR are shown in Figure 3a for E. coli and Figure 3b for S. aureus. All PR values represent average values of five experiments at 11-12 C and 35-37 C and nine experiments at 23-25 C and were normalized to 0.1% survival. E. coli exhibited an initial decrease in PR that persisted for about 15 minutes (lag in PR) for all three temperature ranges. The maximum PR exhibited by E. coli at 23-25 C was 5 times greater than at 35-37 C and 25 times greater than at 11-12 C. The rate of PR was greatest at 23-25 C and least at 11-12 C. Experiments were terminated after 180 minutes exposure at 23-25 C and after 120 minutes exposure at 35-37 C and 11-12 C.

Figure 3b shows the results of the effects of temperature variations on PR of S. aureus. All PR values represent average values of five experiments at 11-12 C and 35-37 C and nine experiments at 23-25 C and were normalized to 0.1% survival. The maximum values exhibited by S. aureus at 23-25 C were 3 times greater than at 11-12 C

Figure 3a. Effect of temperature on PR of Escherichia coli.

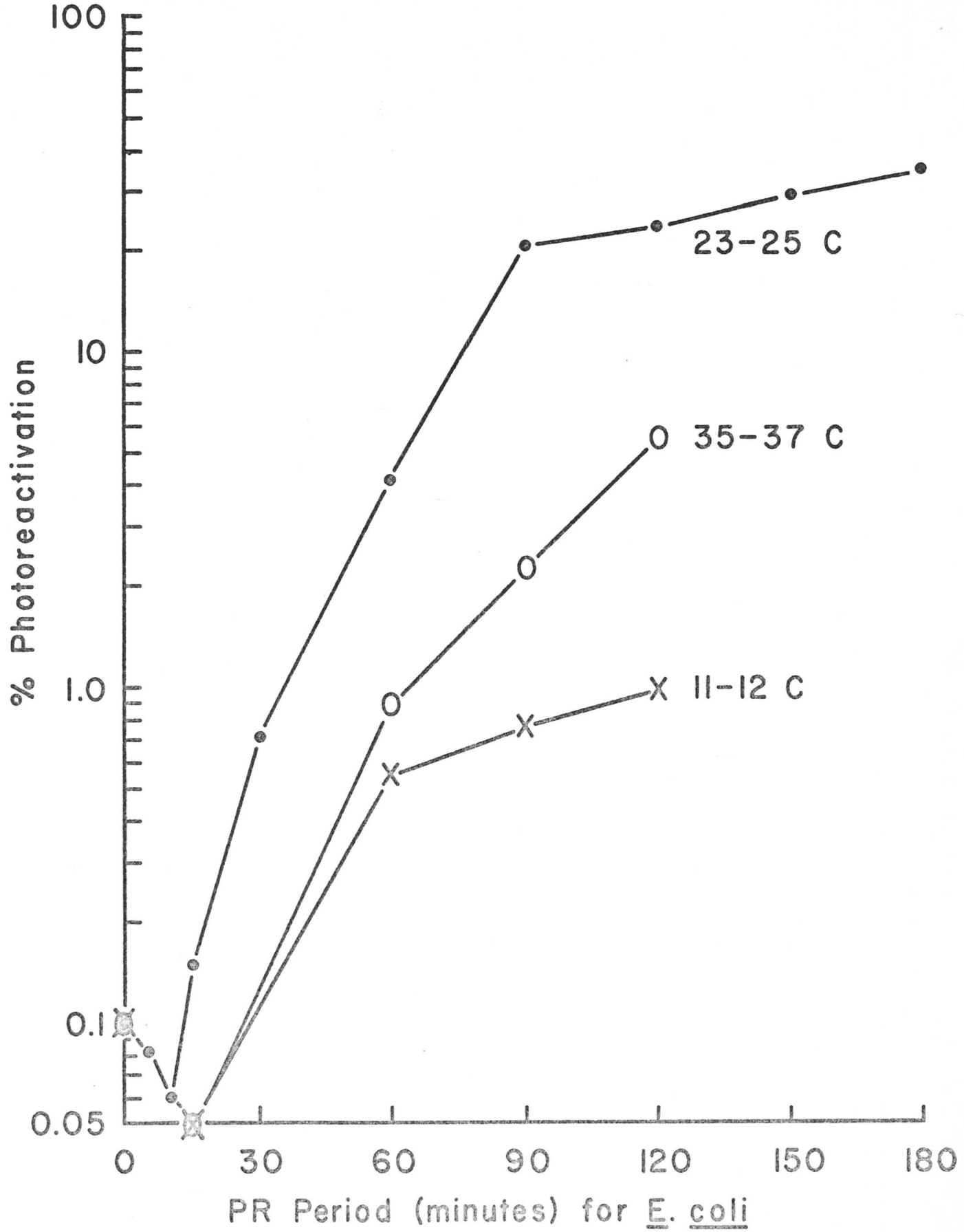
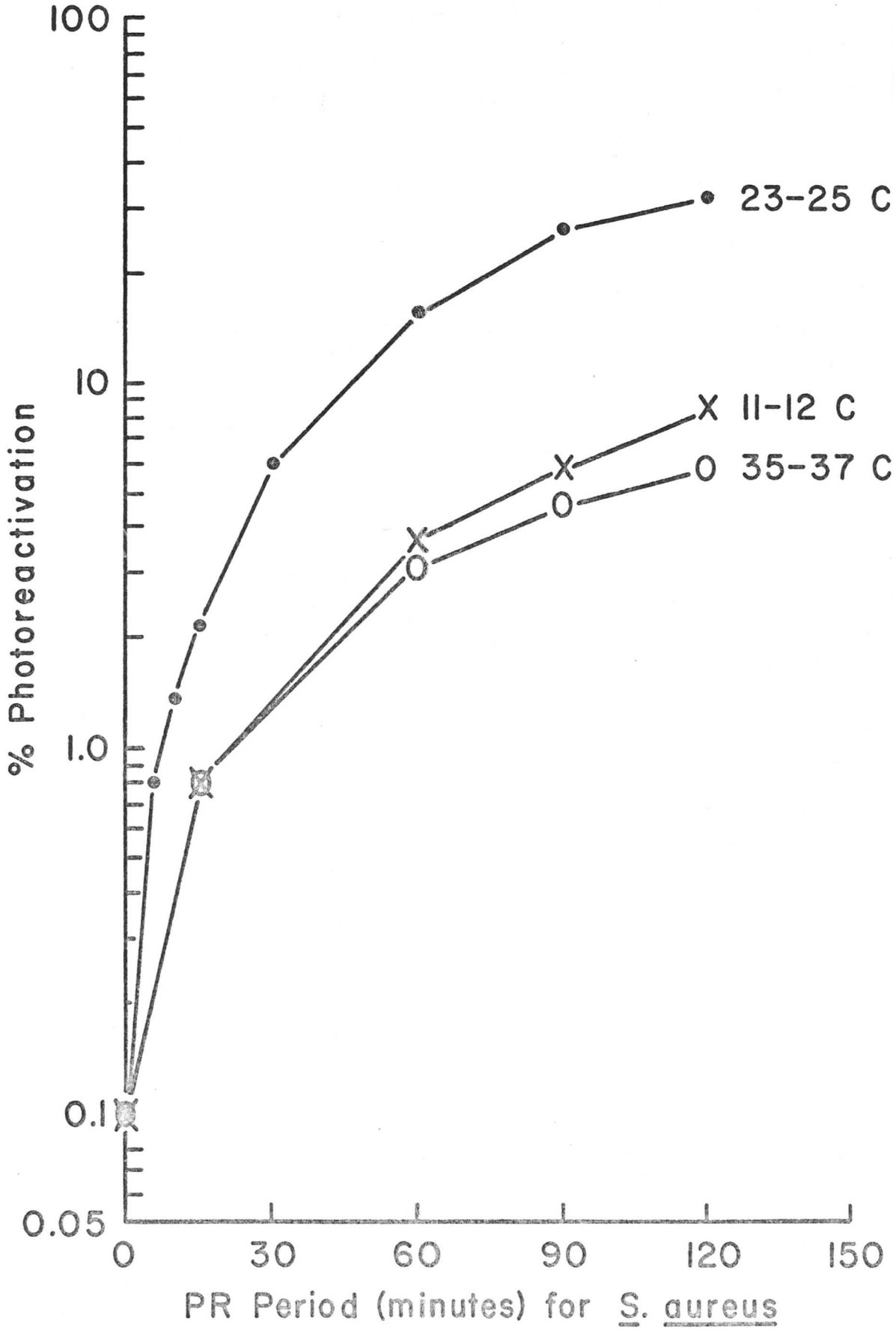

100
10
1.0
0.1
0.05
% Photoreactivation
23-25 C
35-37 C
11-12 C
0
30
60
90
120
150
180
PR Period (minutes) for E. coli

Figure 3b. Effect of temperature on PR of Staphylococcus aureus.


100
10
1.0
0.1
0.05
% Photoreactivation
23-25 C
X 11-12 C
O 35-37 C
0
30
60
90
120
150
PR Period (minutes) for S. aureus

and 6 times greater than at 35-37 C. The rate of PR was greatest at 23-25 C. There was no significant difference in the PR rates at the other temperatures. Experiments were terminated after 120 minutes at all three temperature ranges.

The PR values for both E. coli (Figure 3a) and S. aureus (Figure 3b) were highest at 23-25 C. E. coli (Figure 3a) exhibited an initial lag in PR at all temperatures, but S. aureus (Figure 3b) did not. E. coli (Figure 3a) exhibited approximately the same PR at 35-37 C as S. aureus (Figure 3b) at this temperature. S. aureus (Figure 3b) exhibited 8 times the PR at 11-12 C than E. coli (Figure 3a) at this temperature. The lowest PR values for E. coli (Figure 3a) were observed at 11-12 C and for S. aureus (Figure 3b) at 35-37 C.

The effects of varying light intensity on PR at 23-25 C are given in Table 3. The intensities and dosages given were for white and blue light. All PR values were normalized to 0.1% survival. The PR values for E. coli decreased with the decreases in dosage. The percent PR values for S. aureus decreased with the decreases in dosage, but with no significant decrease between 50% full intensity and 1% full intensity (white light). There was a 20-fold decrease in the % PR for both E. coli and S. aureus upon exposure to one-half the light intensity. The PR values for S. aureus were 4-10 times greater than those for E. coli for all light intensities (white light) except for blue light intensities. S. aureus did not exhibit PR with blue light.

Table 4 shows the effect of pigmentation in S. aureus on PR at 23 C. All PR values were normalized to 0.1% survival. The PR dosage

used for this experiment was 3.31×10^6 ergs/mm^2. Pigmented S. aureus exhibited 25% greater PR capacity than the white (chromogenic mutant) S. aureus. The white S. aureus, however, still had 4 times the PR capacity of E. coli and the pigmented S. aureus had 5.5 times the PR capacity of E. coli.

Table 3. Effect of light intensity on PR of Escherichia coli and Staphylococcus aureus at 23-25 C.

Dosage (Intensity and Color)	% Survivors	% PR (60 minutes)	
		E. coli	S. aureus
3.31×10^6 ergs/mm^2 (Full intensity, White light)	0.1	4.37	15.47
1.62×10^6 ergs/mm^2 (50% Full, White light)	0.1	0.22	0.75
3.60×10^4 ergs/mm^2 (1% Full, White light)	0.1	0.10	1.00
3.60×10^4 ergs/mm^2 (Filtered-full intensity, Blue light)	0.1	0.10	0

Table 4. Effect of pigmentation of Staphylococcus aureus on PR (3.31×10^6 ergs/mm^2) at 23 C.

Organism	% Survivors	% PR (60 minutes)
Pigmented S. aureus	0.1	8.80
White S. aureus	0.1	6.68
E. coli	0.1	1.60

The PR values for S. aureus at 3.31×10^6 ergs/mm^2 in Table 3 were 2 times greater than the PR values for S. aureus at the same intensity in Table 4. E. coli exhibited 4 times the PR in Table 3 as in Table 4 at 3.31×10^6 ergs/mm^2.

The effects of Chloramphenicol and Puromycin on the growth of E. coli and S. aureus at 23 C are given in Table 5. The minimal inhibitory concentration of Chloramphenicol for E. coli and S. aureus was less than 1 µg/ml. The minimal inhibitory concentration of Puromycin for E. coli was 50 µg/ml. The minimal inhibitory concentration of Puromycin for S. aureus was 10 µg/ml.

The results of the effects of Chloramphenicol and Puromycin on PR of E. coli and S. aureus are given in Tables 6a and 6b. All PR values were normalized to 0.1% survival. Table 6a shows the effect of two different concentrations of Chloramphenicol on PR of E. coli and S. aureus at 23-26 C. Both 10 µg/ml-treated samples of E. coli and S. aureus exhibited approximately the same PR as the untreated samples. The 50 µg/ml-treated samples of S. aureus and E. coli exhibited 73 and 107 times less PR than the untreated samples, respectively.

Table 6b shows the effect of Puromycin (50 µg/ml) on PR of E. coli and S. aureus at 25 C. The untreated sample of E. coli exhibited approximately twice as much PR as the treated sample. The treated sample of S. aureus exhibited approximately the same PR as the untreated sample.

The PR values for the untreated samples of E. coli at 3.31×10^6

ergs/mm^2 in Tables 6a and 6b were approximately the same, but were less than the PR values in Figures 2, 3a and 3b and in Table 3. One sample of untreated S. aureus (Table 6a) exhibited approximately 7 times the PR as a similar sample in Table 6a and the untreated sample in Table 6b. These PR values for S. aureus varied from 2 to 25 times less than the PR values reported in earlier figures and tables.

Table 5. Effect of antibiotics on growth of Escherichia coli and Staphylococcus aureus at 23 C.

Antibiotic	Concentration (µg/ml)	Growth*	
		E. coli	S. aureus
Chloramphenicol	500	-	-
	100	-	-
	50	-	-
	10	-	-
	1	-	-
	0 (Control)	+	+
Puromycin	500	-	-
	100	-	-
	50	-	-
	10	+	-
	1	+	+
	0 (Control)	+	+

*(+), growth; (-), no growth.

Table 6a. Effect of Chloramphenicol on PR of Escherichia coli and Staphylococcus aureus at 23-26 C.

Chloramphenicol (µg/ml)	% Survivors	% PR (3.31×10^6 ergs/mm^2)	
		E. coli	S. aureus
0	0.1	0.75	0.85
10	0.1	0.88	1.06
0	0.1	1.07	7.25
50	0.1	0.01	0.09

Table 6b. Effect of Puromycin on PR of Escherichia coli and Staphylococcus aureus at 25 C.

Puromycin (µg/ml)	% Survivors	% PR (3.31×10^6 ergs/mm^2)	
		E. coli	S. aureus
0	0.1	1.61	0.61
50	0.1	0.77	0.84

DISCUSSION

Gates (1928, 1930) reported the action spectra for UV inactivation in bacteria. Jagger (1964) reported that bacteria were generally susceptible to inactivation by low doses (10^1 to 10^4 ergs/mm^2) of UV irradiation. All UV dosages employed in the experiments were within this range. The UV dosage required for 99.9% lethality of E. coli strain B represented 1.8 times the dosage for the same lethality of S. aureus strain 7-8 (Figure 1).

Variation existed in the responses of different cultures of Staphylococcus to UV-irradiation doses. UV irradiation of S. aureus strain 7-8 with 1.75×10^3 ergs/mm^2 was 99.9% lethal (Figure 1). Goering and Pattee (1971) reported UV irradiation with one-third this dosage was 99.5% lethal to S. aureus strain 112. Ikenaga et al. (1970) reported UV irradiation with 6.5×10^2 ergs/mm^2 (approximately one-quarter of the dosage above) was 99% lethal to S. epidermidis.

Results of the effect of UV dosage on delay of growth and division of E. coli strain B were presented by Takebe and Jagger (1969) and Kantor and Deering (1967). Growth and division were inhibited by 31.5 to 157.5 times less dosage than the UV dosage 99.9% lethal for our E. coli strain B (Figure 1). Radiation-resistant E. coli strain B/r was inactivated by UV irradiation with 2.0×10^4 ergs/mm^2 (Ikenaga et al., 1970). This was 6.4 times greater dosage than that proving 99.9% lethal for E. coli strain B (Figure 1). The wavelengths employed in all of the above studies were 230-295 nm.

Results of the survival of E. coli and S. aureus to UV irradiation

(Figure 1) indicated that E. coli was more resistant than S. aureus. Singer and Ames (1970) reported the DNA of Staphylococcus contained approximately 66% adenine (A) plus thymine (T) and the DNA of Escherichia contained approximately 49% A plus T content. Beukers and Berends (1960) reported the primary effect of UV irradiation of DNA was the production of thymine dimers. Subsequent replication of the DNA was halted at the dimer. This was lethal to the cell. These differences in DNA base ratios indicated that S. aureus would be expected to be more sensitive than E. coli to UV irradiation. Assuming that the repair mechanisms were the same for E. coli and S. aureus, then the repair system in S. aureus had more damage to repair. This was indicated by the results observed in Figure 1.

The apparent greater sensitivity of S. aureus to UV irradiation could reflect a less efficient repair capacity for damage due to UV irradiation. The hypothesis that E. coli can live free of a host and might have evolved a more efficient repair mechanism than S. aureus, an internal pathogen rarely exposed to UV irradiation was disproved in later experiments (Figures 2 and 3). The UV resistance exhibited by E. coli might be related to the difference in morphology of these two organisms. Cells of E. coli are rod shaped and approximately twice as large as cells of S. aureus. The orientation of the cells in the UV-irradiated suspension and the position of the DNA within these rods could relate to the UV resistance exhibited by E. coli. Each E. coli cell contained approximately twice as much cytoplasmic content to absorb UV irradiation compared to S. aureus cells.

All PR and LHR values were normalized to 0.1% survival. This procedure was necessary since the % survival values of all experiments varied. The calculations involved in the normalization procedure compensated for LHR that occurred in all PR experiments. All PR values were minus any increase in cell number due to LHR.

S. aureus had a significantly greater capacity than E. coli for repair of UV damage. Both UV irradiation and white light are foreign to the natural habitat of S. aureus. It was possible that the PR capacity of S. aureus observed in these experiments was the manifestation of a mechanism normally utilized for a different purpose by this internal pathogen. It could be proposed that S. aureus has not been an internal parasite for very long. S. aureus would have required a more efficient repair mechanism than E. coli to compensate for the greater effects of UV irradiation to cells with high thymine content in their DNA.

Pigmentation of S. aureus may have permitted greater absorption of photons when compared to non-pigmented E. coli. Different biological pigments absorb various wavelengths of light. Many transmit the light energy to systems capable of its utilization (i.e. accessory plant pigments; Devlin, 1969). This may have accounted for the enhanced rate of PR observed for S. aureus (Figures 2, 3a and 3b) and was substantiated by the lag in PR response observed with the low PR dosages for E. coli (Figures 2 and 3a).

The results of the effect of pigmentation of S. aureus on PR were given in Table 4. The white S. aureus was still pigmented, but being a different color, would be expected to exhibit a difference in photon

absorption when compared to the normally golden-pigmented S. aureus. If pigmentation was a factor involved in light absorption and ultimately the PR capacity, the white mutant would be expected to show a different PR response than the golden S. aureus (Table 4). Alternately, the white mutant culture of S. aureus could possibly be a PR mutant and thus exhibit a different response to the PR light. Either or both of these possibilities may account for the lower PR response of the white mutant when compared to the PR response of normal S. aureus. The total effect of pigment production on PR could not be determined by these experiments. Additional experiments using non-pigmented or "transparent" mutants of S. aureus could provide further clarification of this point.

The % PR obtained for E. coli B in our experiments could not be compared with the results of other workers since either the % PR was not determined in their experiments or the PR dosage was not given. There are no reports in the literature of PR of S. aureus. Our data indicated S. aureus exhibited 0.3 times greater PR than E. coli for the same dosage (Figure 2).

E. coli exhibited LHR capacity over a wide temperature range (11-25 C, Table 2). E. coli exists naturally at all of the experimental temperatures tested. Lethal replication of UV-damaged DNA may have occurred at 35-37 C during the short LHR period and thus accounted for the 0% LHR at that temperature (Table 2). Lethal replication of DNA did not occur during the short LHR period at the lower temperatures used.

The LHR capacity exhibited by S. aureus occurred within a narrowly defined temperature range below optimum temperatures for growth.

Apparently, S. aureus must also be maintained under non-growth conditions which allow time for the repair of the UV-induced damage before the lethal error can be replicated. This accounted for the 0% LHR obtained at 35-37 C. S. aureus normally exists within a narrow temperature range of the host organism and was not capable of LHR at a temperature as low as 11-12 C.

It would be interesting to compare the low-temperature LHR capacity of S. epidermidis, an external parasite, with S. aureus. The LHR capacity of S. epidermidis has not been reported in the literature.

The LHR capacity of both E. coli and S. aureus were measured for 15-60 minutes at 23-25 C (Table 2). S. aureus exhibited approximately the same LHR over this holding period. E. coli exhibited a tenfold increase in % LHR during the 15-60 minutes holding period. These results indicated that E. coli exhibited greater LHR than S. aureus. The relation of this difference in LHR capacity to the survival of these species in Nature is not clear.

Results of the effect of temperature on PR of E. coli and S. aureus were given in Figures 3a and 3b, respectively. The results of 23-25 C temperature effects on PR of E. coli and S. aureus have already been discussed. The maximum PR for both cultures was obtained at 23-25 C. The results indicated that the PR capacity of S. aureus (Figure 3b) was not as temperature dependent as the PR capacity of E. coli (Figure 3a). The PR capacity of both E. coli and S. aureus was less at 11-12 C and 35-37 C than at 23-25 C. This indicated these temperatures were below and above the temperature for maximum PR. The PR capacity of S.

aureus was lowest at the elevated temperature range. The PR capacity of E. coli was lowest at the lowest temperature range. It is improbable that the same enzymatic repair system for E. coli and S. aureus could have such different temperature response curves. The different response may, however, actually reflect a difference in some type of temperature dependent transport or synthetic capacity indirectly affecting the PR capacity.

S. aureus exhibited a narrow temperature range requirement for LHR, but the PR capacity was active over a broad temperature range. These results indicated the LHR and PR systems were different for S. aureus. This was in disagreement with what had been reported for another species. Castellani et al., (1964) reported the LHR and PR systems were apparently identical in E. coli strain B. S. aureus may have lost its LHR capacity at low and high temperatures, but still have PR capacity at these temperatures. Whether or not these results reflect some type of evolutionary trend might be clarified by further investigation of LHR and PR capacities of different cultures of S. aureus to see it this temperature relationship was a constant feature of members of this species.

The data in Table 3 substantiates our above results that showed an enhanced PR capacity of S. aureus over E. coli. The exposure time used was not sufficient for the occurrence of maximum PR of either E. coli or S. aureus. The results in Table 3 show that the PR capacity of E. coli varied directly with the light intensity whereas S. aureus does not demonstrate this direct relationship. The PR capacity of S. aureus was identical at 50% full intensity and at 1% full intensity. The PR

capacity for both E. coli and S. aureus was greatly reduced with the reduced intensity of PR light; however, the failure of S. aureus to undergo additional reduction in PR values with further reduction in light intensity deserves comment. S. aureus seemingly would have enhanced PR capacity, as compared to E. coli, in Nature where limited dosages of light could exist.

Rupert et al. (1971) reported that E. coli exhibited maximum PR within a wavelength range of 355-385 nm. Similarly, Ikenaga et al. (1970) reported that S. epidermidis exhibited from 5 to 9 times greater PR at 313-365 nm (near UV) than at 436 nm (violet-blue light). We predicted reduced or no PR response by E. coli and S. aureus to blue light (450 nm) based upon the above reports. S. aureus did exhibit a lack of PR capacity to the blue light, whereas E. coli did exhibit reduced PR with blue light (Table 3). These response differences indicated that we were in fact observing PR in S. aureus and E. coli. Our experimental results would have been more comparable if we had used a monochromator for the PR light source.

Variations did occur in the PR values for E. coli and S. aureus between Tables 3, 4, 6a and 6b. Our differences in the PR response were not unexpected since variations among identical strains as well as among different strains, species and genera had been previously reported by various workers. In all cases, however, the patterns of differences were consistent. We feel that these variations represented technical differences inherent in the techniques employed (i.e. differences in the number of cells exposed to UV irradiation or differences in the UV-

irradiation period). The first effect may be eliminated by careful adjustment of cell titers to equal numbers of viable cells for both E. coli and S. aureus for all experiments. This was impossible in experiments reported here since actual viable counts were determined after the experiment was completed. The differences in size and shape of cells of these species rendered turbidometric adjustment of cell titers unreliable. This difference in UV-exposure periods can be eliminated by use of a mechanical shutter to exactly time the periods of UV irradiation.

Preliminary experiments were conducted to determine if the ability to produce the enzymes involved in PR were inducible by light or were light-activated constitutive enzymes. The action of two antibiotics affecting protein synthesis on photoenzymatic repair by E. coli and S. aureus were presented in Tables 6a and 6b. Hahn et al. (1953), Brock (1961), and Jardetzky (1962) reviewed the effect of Chloramphenicol on bacteria. Chloramphenicol reportedly inhibited protein synthesis by binding with the 50S ribosomal subunit and interferring with peptide-forming steps. Nathans (1964), Traut and Monro (1964), and Tanaka et al. (1972) reviewed the effect of Puromycin on bacteria. Puromycin reportedly inhibited protein synthesis by inhibiting peptide-chain elongation. This was effected by substitution of Puromycin for an aminoacyl-transfer-RNA resulting in the formation of a peptidyl-puromycin derivative on the ribosome and the cessation of polypeptide synthesis. These incomplete polypeptides had no enzymatic activity.

The concentrations of Chloramphenicol and Puromycin inhibiting the growth of E. coli and S. aureus were determined (Table 5) and the

effect of the presence of each during the PR period determined (Tables 6a and 6b). There was no significant difference in PR values for S. aureus or E. coli treated with 10 µg/ml Chloramphenicol compared to PR values of untreated samples. These results indicated that the growth of cells in 10 µg/ml Chloramphenicol inhibited synthesis of enzymes required for cell division but treatment of cells with 10 µg/ml Chloramphenicol during the PR period did not produce any decrease in the PR response of either culture. If the exposure to white light induced the synthesis of photoenzymatic repair enzymes by the cell and if 10 µg/ml Chloramphenicol inhibited the synthesis of all enzymes during the PR period, then these results indicated the photoenzymatic repair enzymes were constitutive to the cell prior to white light and antibiotic exposure.

Various explanations could account for these results. One-hour exposure of cells to 10 µg/ml Chloramphenicol followed by dilution on growth medium might not have inhibited growth or the synthesis of any enzymes. The experiment was repeated using Puromycin, since inhibition of protein synthesis by this drug would not be easily reversed by dilution. The comparatively non-reversible inhibition of protein synthesis by Puromycin had been previously reported by Nathans (1964).

Cells irradiated with UV and exposed to Puromycin during PR would be expected to show greatly decreased PR values if the enzymes producing PR were inducible. There was no great effect on the PR of S. aureus after comparing the results of treated and untreated samples (Table 6b). This indicated Puromycin did not have an effect on the photoenzymatic

response of those cells surviving any lethal effect (if treatment with 50 µg/ml Puromycin during the PR period caused growth inhibition by inhibiting protein synthesis). This substantiated the results obtained above with Chloramphenicol. Treatment with 50 µg/ml Puromycin produced approximately 50% reduction in PR values for E. coli. This could indicate E. coli was more sensitive than S. aureus to the action of Puromycin or that the Puromycin effect was able to be reversed by S. aureus but not by E. coli. The relationship of Puromycin to enzyme inhibition or induction was not clear.

Exposure of the cultures to a high concentration of Chloramphenicol during the PR period should result in a great decrease in PR values if cells are inhibited by the drug. Results of treatment with a high concentration of Chloramphenicol should clarify difficulties indicated above in the interpretation of the actual effect of the antibiotics (i.e. whether growth inhibition, PR inhibition, or both). Any cells showing a response would likely be those recovering from UV damage and drug inhibition. Any photoenzymatic recovery upon exposure to 50-times the growth-inhibiting dose would indicate the constitutive nature of the photoenzymes. This experiment would also clarify the proposed reversal of the Chloramphenicol effect by dilution upon plating. Any dilution would have to be five times more effective than in the previous experiment with this drug to cause similar reversal. Treatment with 50 µg/ml Chloramphenicol produced a great decrease in PR values for E. coli and S. aureus (Table 6a). The persistence of photoenzymatic recovery upon treatment for one hour with over fifty times the dose of

Chloramphenicol inhibiting cell division suggested the enzymes instrumental in PR were already present in the cell at the time of exposure to the PR conditions and the drug.

The preliminary nature of these inhibition experiments was indicated above. Several variables and alternate explanations exist which require further investigation before the effect of these drugs on the PR response can be clarified.

SUMMARY

The capacity of Staphylococcus aureus strain 7-8 to undergo photoenzymatic repair of UV-irradiation induced damage was compared to the photoreactivation (PR) response of Escherichia coli strain B. Staphylococcus aureus showed greater inhibition by UV irradiation than E. coli. This was consistent with the higher adenine and thymine content of S. aureus. Staphylococcus aureus showed an enhanced rate of photoreactivation with no lag in initiation of the PR response with low PR doses compared to E. coli. Maximum PR capacity of both cultures was about equal and occurred for cultures incubated at 23-25 C. The PR responses at 11-12 C and 35-37 C for S. aureus and E. coli differed, although both were capable of PR at all temperatures employed. The PR response of E. coli was directly related to the dosage of PR light (ergs/mm^2); however, the photoenzymatic capacity of S. aureus was not directly responsive to continued decrease in light intensity. The capacity of S. aureus to undergo liquid holding recovery (LHR) occurred within a narrow temperature range (23-25 C), whereas E. coli underwent LHR at all but the highest temperatures (35-37 C). The LHR response of S. aureus was somewhat more effective than E. coli and did not show the direct response to increased liquid-holding period as E. coli.

The PR value of a white pigmentation mutant of S. aureus was different from the normally pigmented parent. The relation of pigment production to photoenzymatic response of S. aureus requires further clarification.

The PR values were non-refractory to exposure of cells to PR

conditions in the presence of antibiotics inhibiting protein synthesis. These preliminaty results may indicate the constitutive occurrence of PR enzymes.

The differences observed in PR capacity and LHR response of E. coli and S. aureus to conditions of PR exposure and pigmentation were related to the natural habitats of these species.

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