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Drug Modification Of Post-Ultraviolet Survival In Dna Dark-Repair Mutants Of Escherichia Coli.

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DRUG MODIFICATION OF POST-ULTRAVIOLET SURVIVAL IN DNA DARK REPAIR MUTANTS OF *ESCHERICHIA COLI*

by

JOHN CHRISTOPHER LANKFORD, III

A DISSERTATION

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1972

DEDICATION

To my wife, Marion S. Lankford, whose patience, understanding and love inspired this dissertation.

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I. INTRODUCTION

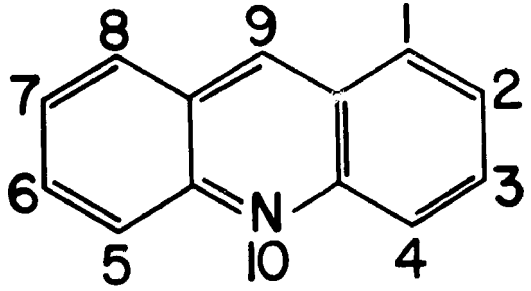
Statement of the Problem

Acridines (Figure 1) bind extensively to DNA and have been used classically as stains for nucleic acids (2). The binding (9,10,29) and mutagenic properties (7) of this class of compounds provide an excellent means of studying the relationship between these properties and regulation of DNA repair mechanisms. Lerman (27) has postulated that these compounds interact with DNA through intercalation, and also demonstrated that methylation of the nitrogen in the quinacrine ring renders the acridine non-mutagenic (28). Riva (37) reported that both mutagenic and non-mutagenic acridines bind to DNA to the same extent. The mechanism for mutagenicity by acridines, however, was still not explained. The discovery of a dependence of mutagenicity on inhibition of dark repair might elucidate the action of acridines.

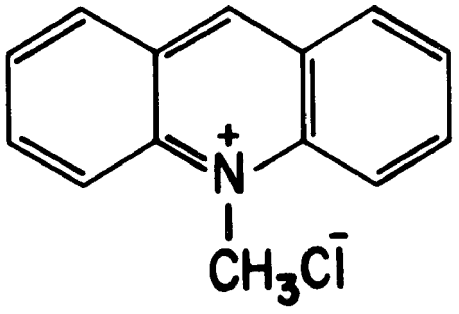
The purpose of this dissertation is to investigate the effect of both mutagenic and non-mutagenic acridines on post-irradiation survival in Escherichia coli B. Subsequently, the specific process being inhibited by the drug should be found, and the ultimate goal is to determine if there is a correlation between acridine mutagenicity and the drug's ability to inhibit dark repair. Thus, various dark

FIGURE 1

THE CHEMICAL STRUCTURES OF VARIOUS ACRIDINES



Acridine



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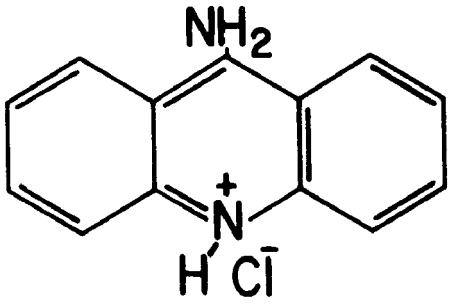

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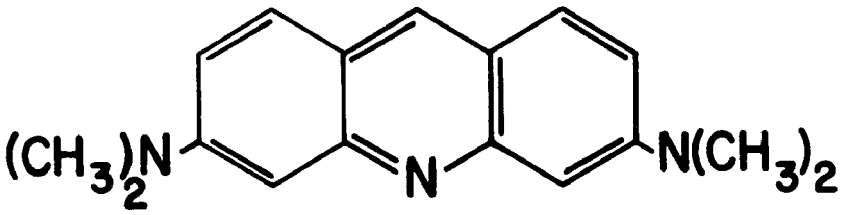


Acridine Orange
3,6- Bisdimethylamino - acridine

repair mutants, such as E. coli B_{s-1} and E. coli B/r were used to discover if dark repair was the process being inhibited by the acridines. If it can be established that dark repair is the process being inhibited by the drug, then it would be informative to know if acridines influence a single enzymic step in the DNA dark repair process rather than exerting an inhibition on the overall repair process.

Mutations

Mutations are among the most important biological phenomena, because the understanding of these events could provide the answers to such problems as aging, cancer, etc. They are the result of discontinuous events which lead to alterations in the genetic apparatus. Since the phenomenon of mutations was first emphasized in the early 1900's, the analysis of this process has occupied a central role in the field of genetics. Mutations are the source of modified genes which serve as the raw material for evolution. These modified genes, in a myriad of combinations, acquired by such processes as replication, recombination, transcription and mutation, are subject to natural selection. The onset of malignant growth, the outbreak of epidemics and hereditary diseases may derive their roots from changes brought about by mutations. The recognition of mutations as visible physical manifestations, e.g., flower color,

and later in a more chemical sense as changes in the sequence of bases in DNA, has had far reaching implications extending into the very basis of nature.

Mutations are divided into two catagories: 1) spontaneous and 2) induced. Spontaneous mutations are the result of the action of unknown events, and may also be considered to be induced even if the mechanism for their appearance is not known. Induced mutations may be further separated into two classes: a) those caused by physical action, such as, ultraviolet light striking DNA and causing thymine dimer formation between two adjacent thymine residues (42), and b) those resulting from the reaction of certain chemical compounds with DNA, e.g., nitrogen mustard, a commonly used mutagen, which causes interchain crosslinking, thereby effectively blocking normal DNA replication.

DNA Repair

DNA, the primary genetic material, serves as the substrate for five primary biological processes. These are mutation, replication, recombination, transcription and repair. Mutation alters the genetic apparatus. Replication duplicates the DNA molecule prior to cell division. Transcription of m-RNA is the first step in the synthesis of proteins. Recombination, which introduces variations into the DNA sequence within a species, is the "crossing over"

of short segments of DNA strands. Repair serves as a homeostatic mechanism capable of correcting aberrations induced in the DNA molecule by physical and chemical agents. However, under certain conditions this same repair system introduces errors into the DNA sequence causing mutations which may be lethal. Actually, all of the processes mentioned, given appropriate conditions, could introduce errors into the DNA sequence, and therefore, could be mutagenic (20,54).

At this point, it would be useful to discuss briefly the three accepted modes of repair used by microorganisms to maintain their normal genetic complement (53). Photoreactivation, the first type of repair, uses a single enzyme to repair thymine dimers caused by ultraviolet light (24,39). The second type of repair was first referred to as "bypass" or post-replicative repair and is now known as recombinational repair (43). In this case, during DNA replication the lesion is bypassed without being repaired. After cell division, the lesion is then corrected using recombinational mechanisms. The third process is known as excision repair or dark repair, because it occurs in the absence of light and involves four different enzymic steps or reactions (5,18,36,40,41). Excision repair is the major form of repair reported in mammals. The first step involves recognition of the damage and incision by an endonuclease through the phosphodiester backbone of the DNA molecule.

This step is followed by the excision process (exonuclease) which removes the damaged bases and several adjacent bases on either side of the damaged ones. This leaves a gap in one strand of the DNA molecule about five or six bases long. Subsequently, a repair polymerase fills in the gap with new bases which are complementary to the opposing chain. Lastly, a ligase seals the gap resulting in the restoration of the normal sequence of the DNA molecule.

Mutants

Microorganisms provide an excellent tool for the study of biological processes, because their rapid turnover allows for the examination of many generations of organisms in a short period of time. Results from these types of studies provide insight into similar phenomena in more complex organisms, e.g., mammals. Mutant forms of bacteria provide an array of organisms which are identical to the wild strain except for one alteration in physiological or biochemical function. Thus, bacterial studies on all three types of repair may provide additional information pertaining to the biological maintenance of the gene integrity.

In order to learn more about the normal role of DNA repair and the regulation of repair enzymes, particularly in relation to mutations, the effect of acridines on dark repair in *E. coli* was studied. Specifically, *E. coli* B was chosen, because it was the wild type

organism and exemplified normal dark repair performance. This organism grows normally on minimal media utilizing glucose as its sole carbon source at 37°C. Also, mutants of E. coli B were chosen for study, because they exhibited extremes in DNA repair response to UV irradiation. E. coli B_{s-1} is a spontaneous mutant which lacks the incision step of DNA dark repair, and thus, demonstrates extreme UV sensitivity because of its inability to initiate dark repair. Since E. coli B_{s-1} possesses normal DNA replication, photoreactivation repair and recombinational repair, this organism could reveal whether these processes were participating in post-ultraviolet survival, and whether specifically dark repair was the process being studied. E. coli B/r is a highly UV resistant mutant which is capable of repairing massive doses of UV irradiation. In order to further support the theory that dark repair is the process being inhibited, this organism was studied. The growth requirements of E. coli B_{s-1} and E. coli B/r are identical to E. coli B.

E. coli ts-7 is a mutant derived from E. coli TAU-bar which produces a temperature-sensitive ligase. Thus, following UV irradiation, the organism displays normal ligase function when grown at 25°C, but demonstrates deficient ligase activity when incubated at 40°C for two hours. This organism's mutated ligase function made the investigation of the inhibition of a single enzymic step in the dark repair possible. This mutant is a polyauxotroph requiring

the addition of arginine, thymine, uracil, proline, tryptophan and methionine to minimal medium. E. coli TAU-bar grows normally at 25°C with the same nutrient requirements as E. coli ts-7. This organism displays normal ligase activity at both 25°C and 40°C, although at 40°C, its post-UV irradiation survival is greater than at 25°C. This mutant was studied in order to compare its post-irradiation survival responses to that of E. coli ts-7.

Acridines

The precise mechanism for binding by these drugs is still a subject for investigation, although it is now generally accepted that they bind at least partially by intercalation (26,28). As originally proposed by Lerman (27), intercalation is the interaction of the π electrons in the quinacrine ring of the acridine with the π electrons in the DNA bases. Intercalation may be pictured as a "sandwiching" of the acridine ring system between the stacked DNA bases. Another type of binding which has been reported (1) is a charge-charge interaction and probably involves the charged acridine ring nitrogen with the charged DNA phosphate backbone. However, it is still not clear which type of binding is related to the biological properties of the drug.

Brenner et al, (6) proposed that mutations resulted if acridines were intercalated and thus sandwiched between nucleotide bases,

causing insertion or deletion of DNA bases. These studies led to the recognition that the different amino acids used in protein synthesis are coded by a triplet of bases in the messenger-RNA (11). Since messenger-RNA is read three bases at a time, the insertion or deletion of a base causes a frameshift in the reading sequence. This misreading is expressed in either amino acid substitutions or chain-terminating codons. These mutations may be lethal to the organism.

Lerman demonstrated that methylation of the nitrogen atom in the quinacrine ring rendered the acridine non-mutagenic (28). Witkin (50) observed that acriflavine had a deleterious effect on the post-ultraviolet irradiation survival of a tyrosine-requiring auxotroph of E. coli B/r. However, since acriflavine is a mixture of a methylated acridine and a non-methylated acridine, these findings were not completely definitive as to whether the pure methylated acridine was mutagenic. Riva (37) studied the binding affinities of some methylated and non-methylated acridines for DNA, and concluded that both types of acridines were bound to the same extent. These results presented the question, "Why does methylation of the acridine nitrogen alter the drug mutagenic properties but not the binding properties?" One possible explanation is that mutagenicity is not the direct result of a specific acridine-DNA interaction, but instead is due to the inhibition of an enzymatic

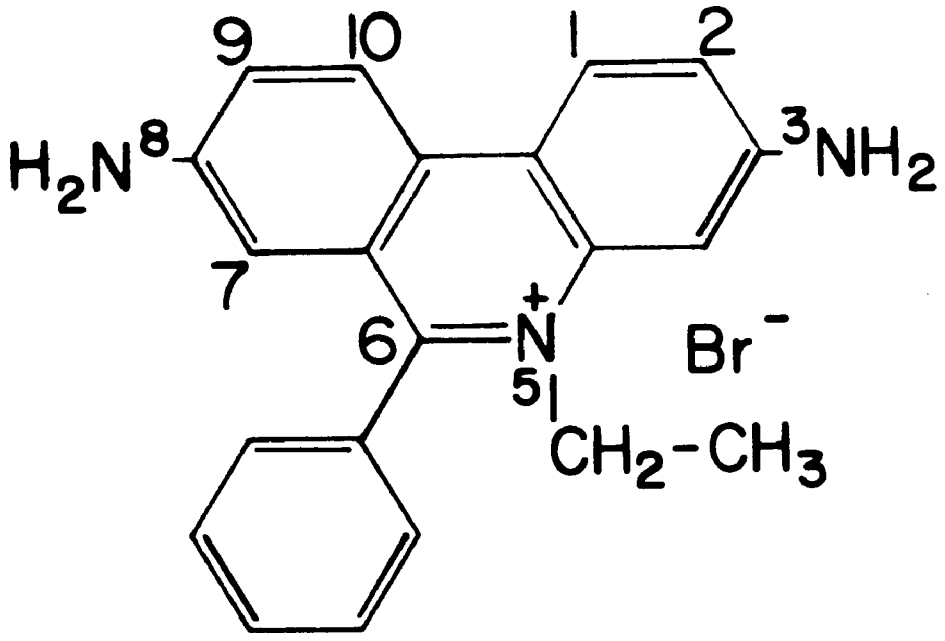
process in DNA repair. Another possibility is that the methyl group allows the acridine-DNA complex to undergo repair normally.

This theory could be tested by determining if there is a relationship between mutagenicity and ability to inhibit DNA dark repair. If there is a correlation, then mutagenic acridines should inhibit dark repair and non-mutagenic ones should not. The initial investigation involved determining the effectiveness of mutagenic (9-aminoacridine hydrochloride and acridine orange) and non-mutagenic (10-methylacridinium chloride) acridines on the post-ultraviolet irradiation survival of E. coli B. Ethidium bromide (Figure 2) was used as a control compound, since it has been shown to be mutagenic in yeast (44), and its mode of interaction with DNA is intercalation (4). These preliminary studies have been published (25).

If it could be shown that mutagenic acridines inhibited repair and non-mutagenic ones did not, then new insight into the process of acridine induction of mutations would result. An exciting possibility is that acridine inhibits DNA dark repair which causes errors in base substitutions resulting in a frameshift mutation. This theory alters the role of the acridines proposed earlier (6) in which they supposedly caused frameshift mutations by intercalation during replication. The fact that several acridines have been shown to bind to DNA with the same strength, suggests the same mechanism of interaction, i.e., intercalation. However, since

FIGURE 2

THE CHEMICAL STRUCTURE OF ETHIDIUM BROMIDE



Ethidium Bromide

all acridines are not mutagenic, it may be concluded that mutagenicity may not simply be a function of intercalation.

Frameshift Mutations

Frameshift mutations as they occur in microorganisms have been explained by three models: 1) errors in replication (3), 2) errors in recombination due to unequal crossing over (27), and 3) errors in DNA repair (46). Each of these models assumes that acridines induce frameshift mutations by intercalation between the bases of DNA, producing miscopying errors (6). The following discussion will be limited to a brief literature review of the first two models and then to a more detailed description of how acridines contribute to the third model.

Considering the first model, Brenner et al, (7) proposed that errors are introduced into the DNA sequence during the process of replication. However, Drake (15) demonstrated that base analogs inhibit T_4 DNA synthesis and reduce proflavin mutagenesis slightly. These findings combined with those of Streisinger (46) and Drake (16) suggested that frameshift mutations clearly add or delete two or more bases making it highly unlikely that frameshift mutations result from simple errors in DNA replication. The results of Newton et al, (33) support the model that acridine-induced mutagenesis is associated with DNA replication. They studied mutants

lacking intact recombination and dark repair systems and found that these organisms were able to revert frameshifts induced by acridines.

Lerman (27) proposed the second model, in which acridines induce unequal recombination by forcing mistakes in homologous base pairing. His model fails to clearly explain the variable number of base pairs added or deleted in frameshift mutations, and it is hard to understand in terms of other models for recombination which suppose that homologous pairing first involves single stranded and then hybrid intermediates. However, some impressive correlations have been established between frameshift mutation and recombination.

Frameshift mutations occur spontaneously in yeast and can be induced by acridines during meiosis (30,31). Also, the mutants are highly recombinant for outside markers regardless of when they arise. Proflavin has been shown to be much more mutagenic during conjugation in E. coli with mutations arising in the vicinity of the recombinational events. This evidence favors Lerman's idea that frameshift mutation results because of errors in recombination. This model is not supported, however, by the fact that no relationship between conjugation and mutagenesis has been observed in Bacillus subtilis (45).

In the third model, Streisinger (46) proposes that acridines act by inducing frameshift mutations by interfering with DNA repair. Specifically there is a mispairing of bases during the repair of

single strand interruptions in the double-stranded DNA molecule. Both addition and deletion mutations are generated, and Inouye et al, (23) and Tsugita (48) have presented specific models for specific frameshift mutations.

The Streisinger model successfully explains a number of the aspects of frameshift mutation. Frameshift mutations are the consequence of breaks in the DNA caused when the incision step of dark repair is evoked by UV irradiation.

The role of acridines in frameshift mutagenesis as described by Streisinger is to stabilize the mispairing configurations in DNA, and thereby increase the chance of dark repair converting the mispaired configuration into a heritable lesion. The suppression of mutation in bacteriophage T_4 at high acridine concentration (14) and suppression of mutation in yeast at low acridine concentration might be related to a more general inhibition of dark repair by acridines. Witkin (51) observed the general inhibition of acridines on the release of thymine dimers induced by UV irradiation. A second point of contradiction of this model is that any compound, regardless of its mode of interaction, should induce frameshift mutations if it stabilizes the double stranded DNA against thermal denaturation. Streptomycin, spermine and magnesium are examples of compounds which stabilize DNA against heat denaturation, but even at moderately high concentrations, they are unable to revert

frameshift mutations. Thus, there appears to be no correlation between mutagenicity and the degree to which compounds stabilize DNA against thermal denaturation. As mentioned earlier, Riva (37) observed no correlation between strength of binding of acridines and their reported mutagenicity. Thus, from the previous studies, there is no clear basis for rationalizing mutagenicity simply in terms of the proposed interactions of drugs with DNA.

A more complete rationale for the role of acridines in mutagenesis may be that of enzyme inhibition resulting from the drug-enzyme interaction, and this is a more discriminating property than simply drug-DNA binding. Thus, the drugs have two distinct molecular properties: one which promotes DNA interaction, and an additional one which leads to enzyme inhibition.

The acridine-DNA complex may be normally "active" with respect to the repair enzymes in the case of the non-mutagenic drugs, i.e., the DNA damage may be repaired as if the acridine had never complexed the DNA. The mutagenic compounds may exhibit an additional type of interaction with the repair enzymes or with the enzyme-DNA complexes, and thus prevent repair. Consequently, the errors would not be repaired, and frameshift mutations would be propagated. Alternately, the drug may provoke drastic lethal errors in repair.

II. MATERIALS AND METHODS

Escherichia coli B

Cultures of E. coli B were grown to late log phase in BHI (Brain Heart Infusion broth, 37g in 1000 ml distilled water) at 37°C in a shaking water bath, and diluted to the appropriate concentration with 0.9% NaCl. The bacteria in a volume of 20 ml were then irradiated in an open petri dish at a distance of 6 inches using a filtered GE G8T5 lamp so that the emitted radiation was 254 mμ. The ultraviolet dose was measured with a Blak-Ray Short Wave UV meter.

Immediately after irradiation 0.1 ml samples of the appropriate dilutions were spread on 1.5% agar plates containing either minimal salts or 3.7% BHI. The minimal salts medium contained 1.0g NH_4Cl, 5.0g NaCl, 6.0g Na_2HPO_4, 3.0g KH_2PO_4, 0.1g $MgSO_4$, and 5.0g glucose per liter (41). The acridine analogs were incorporated in the media at concentrations ranging from 1.0×10^{-6} to 1.0×10^{-4} M. Care was taken to keep the organisms in the dark after irradiation to prevent photoreactivation repair, and incubation was conducted in the dark at 37°C. Bacteria on minimal-agar plates were incubated approximately 40 hours while those on BHI were

incubated overnight. The surviving colonies were counted on a New Brunswick Counter. Each curve represents the average of two or three experiments and the experimental points on each curve represent the average total counts of four plates (32).

DNA Repair Mutants

Individual cultures of E. coli ts-7 and E. coli TAU-bar were grown to mid-log phase in Tryptone broth containing 10.0g Bacto-Tryptone, and 5.0g NaCl per liter (35). Irradiation and plating were as described previously. Immediately following plating half of the plates were incubated in the dark at 40°C for 2 hours. Subsequently, they were incubated with the other organisms at 25°C for 24-36 hours or until the colonies were large enough to be counted. Colonies were counted on a New Brunswick Counter.

Culture methods for E. coli B_{s-1} and E. coli B/r were the same as those used for E. coli B, using BHI media.

A list of the various chemicals, organisms and instruments and their sources are enumerated in the Table on Experimental Materials.

TABLE I

Experimental Materials

Brain Heart Infusion Media	Difco Laboratories Detroit, Michigan
Bacto-Tryptone	Difco Laboratories Detroit, Michigan
Escherichia coli B - wild type	American Type Culture Collection Rockville, Maryland
Escherichia coli TAU-bar - parent strain of *E. coli* ts-7; polyauxotrophic, requiring uracil, thymine, arginine, proline, methionine and tryptophan	Dr. Crellin Pauling University of California Riverside, California
Escherichia coli ts-7 - temperature-sensitive ligase mutant; growth requirements same as *E. coli* TAU-bar	Dr. Crellin Pauling University of California Riverside, California
Escherichia coli B_{s-1} - UV sensitive mutant lacking incision enzyme for repair	Biology Division Oak Ridge National Laboratory Oak Ridge, Tennessee
Escherichia coli B/r - UV resistant mutant	Biology Division Oak Ridge National Laboratory Oak Ridge, Tennessee
9-Aminoacridine Hydrochloride	Aldrich Chemical Company, Inc. Milwaukee, Wisconsin
10-Methylacridinium Chloride	Eastman Organic Chemicals Rochester, New York
Acridine Orange	Eastman Organic Chemicals Rochester, New York

Ethidium Bromide	Calbiochem Atlanta, Georgia
Blak-Ray Short Wave UV meter	Ultra-Violet Products, Inc. San Gabriel, California
UV Germicidal Lamp	George Gates and Company Franklin Square L. I., N. Y.
GE G8T5 Lamp	George Gates and Company Franklin Square L. I., N. Y.
New Brunswick Colony Counter	New Brunswick Scientific Company New Brunswick, New Jersey

III. RESULTS

Drug Response Studies

Acridines and ethidium have been used extensively in the treatment of parasitic infections (2,8), and their cytotoxicity is well established. In order to study in detail the effects of the various drugs on DNA repair in E. coli, it was necessary to determine concentrations which were non-lethal to the organisms and would permit repair of moderate UV damage to the DNA. Thus, the effect of drug concentration was determined on the survival of organisms irradiated with a UV dose of 50 ergs/mm^2, an irradiation level which by itself reduced survival by about 40%.

For these experiments E. coli B irradiated with a single UV dose of 50 ergs/mm^2 were plated on minimal agar containing the different drugs at concentrations varying from 1.0×10^{-7} to 3.0×10^{-6} M. The survival response of E. coli B is shown in Figure 3 using 10-methylacridinium chloride (Curve A), acridine orange (Curve B), 9-aminoacridine hydrochloride (Curve C) and ethidium bromide (Curve D). At concentrations greater than 1.0×10^{-6} M, ethidium bromide and 9-aminoacridine hydrochloride were lethal to the organisms, while 10-methylacridinium chloride, the non-mutagenic acridine,

FIGURE 3

THE DRUG RESPONSE CURVES OF E. COLI B TO THE VARIOUS ACRIDINES AND ETHIDIUM BROMIDE

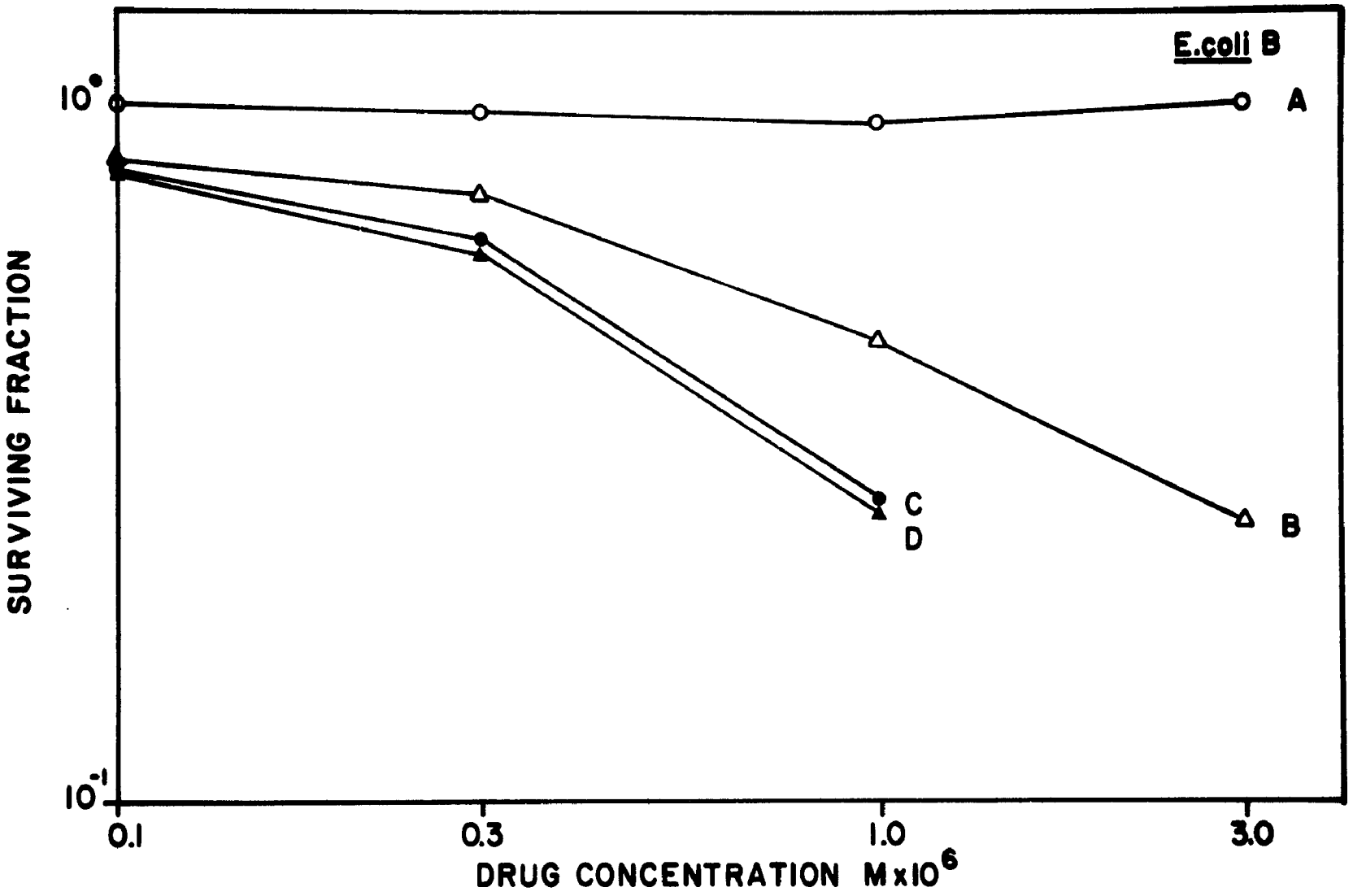
After a single UV exposure of 50 ergs/mm^2, the organisms were spread on minimal agar containing the drugs at concentrations varying from 1.0×10^{-7} to 3.0×10^{-6} M. The plates were then incubated in the dark at 37°C for approximately 36 hours.

Curve A - 10-methylacridinium chloride

Curve B - acridine orange

Curve C - 9-aminoacridine hydrochloride

Curve D - ethidium bromide


E.coli B
A
B
C
D
SURVIVING FRACTION
10⁰
10⁻¹
0.1
0.3
1.0
3.0
DRUG CONCENTRATION M x 10⁶

exhibited a complete lack of drug toxicity over the entire concentration range. Acridine orange was not toxic at a concentration of 3.0×10^{-6} M. E. coli B_s-1 and E. coli B/r had growth requirements identical to E. coli B and, therefore, their post-irradiation survival was studied at the same drug concentration as E. coli B.

E. coli ts-7, a temperature-sensitive ligase mutant, was cultured on Tryptone agar containing 10-methylacridinium chloride and 9-aminoacridine hydrochloride with a concentration range of 1.0×10^{-6} to 1.0×10^{-4} M. The effects of 9-aminoacridine hydrochloride concentration on the viability of E. coli ts-7 are shown in Figure 4. Curves A and C represent the unirradiated controls incubated at 25°C and 40°C, respectively. Curves B and D demonstrate survival after a UV dose of 50 ergs/mm^2 at 25°C and 40°C, respectively, as a function of the concentration of the drug. Although this mutant at 25°C showed viability at 3.0×10^{-5} M 9-aminoacridine hydrochloride, it failed to grow at 40°C. Thus, this organism was more resistant to the drug, and concentrations of 1.0×10^{-5} M were required for the ultimate study of inhibition of its post-irradiation survival. However, since 1.0×10^{-6} M 9-aminoacridine hydrochloride was effective in inhibiting post-irradiation survival of E. coli B, this concentration was also tested at a variety of UV exposure levels in E. coli ts-7 and found to be without effect.

FIGURE 4

THE DRUG RESPONSE CURVES OF E. COLI ts-7
TO 9-AMINOACRIDINE HYDROCHLORIDE

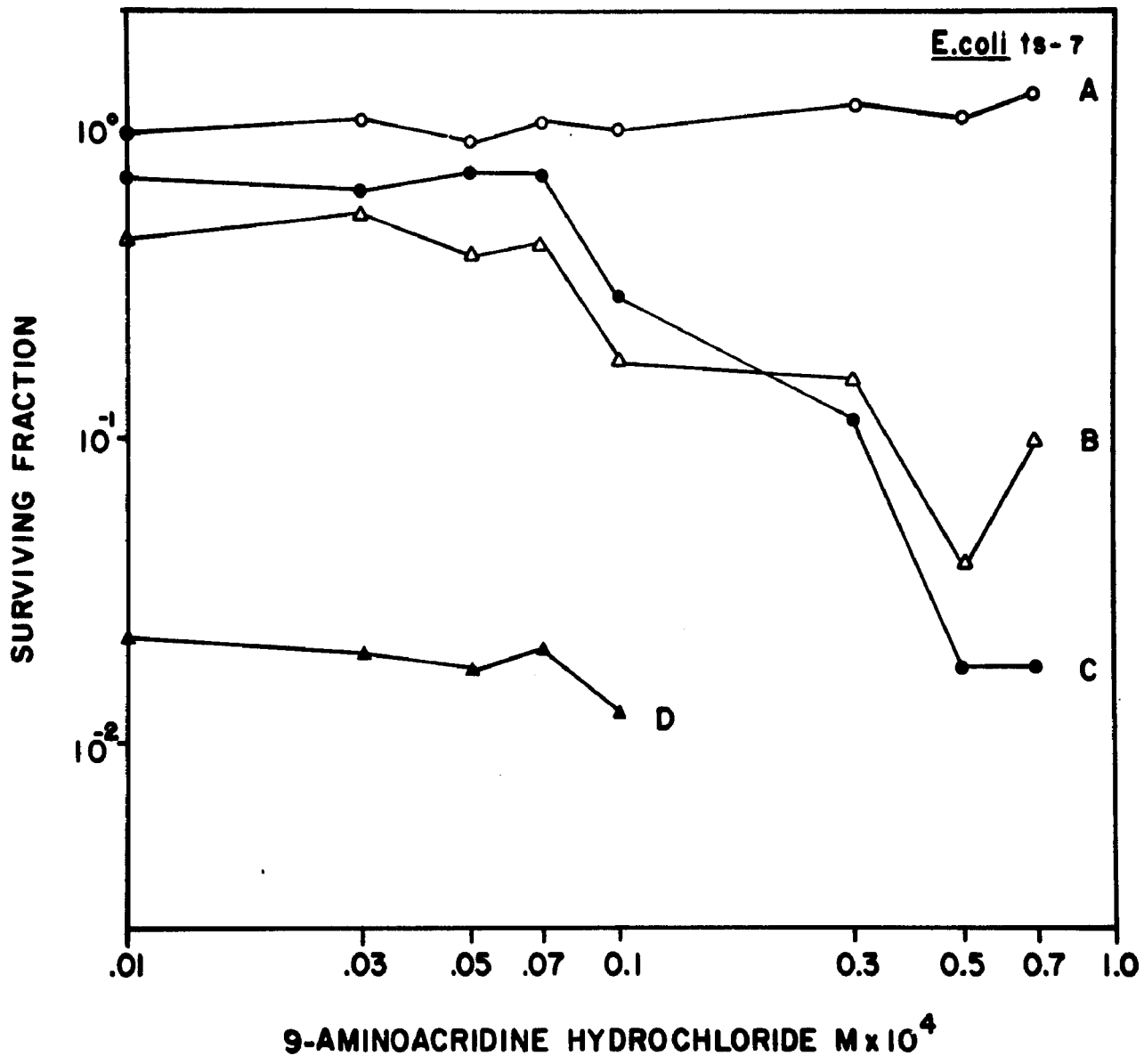
Control organisms were plated without UV irradiation. The remaining organisms received a single UV exposure of 50 ergs/mm^2. Following irradiation, the organisms were spread on Tryptone agar containing various concentrations of the drug. The plates were separated into two groups. One group of plates was incubated in the dark at 40°C for 2 hours, and then placed with the other group which had been incubated in the dark at 25°C. The total incubation for both groups of plates at 25°C was 24-36 hours.

Curve A - organisms grown at 25°C without UV irradiation.

Curve B - organisms grown at 40°C without UV irradiation.

Curve C - organisms grown at 25°C with UV irradiation

Curve D - organisms grown at 40°C with UV irradiation.


E.coli ts-7
A
B
C
D
10^{0}
10^{-1}
10^{-2}
SURVIVING FRACTION
.01
.03
.05
.07
0.1
0.3
0.5
0.7
1.0
9-AMINOACRIDINE HYDROCHLORIDE M x 10^{4}

The drug survival response of E. coli ts-7 to the non-mutagen, 10-methylacridinium chloride is shown in Figure 5. Control Curves A and C represent survival of these organisms at 25°C and 40°C, respectively, in the absence of UV irradiation. Curves B and D reflect survival at 25°C and 40°C, respectively, after a UV dose of 50 ergs/mm^2. The results showed the drug to be non-lethal for either irradiated or non-irradiated organisms over the entire concentration range, as was the case with E. coli B.

E. coli B

Experiments were then performed on the post-irradiation survival of E. coli B using fixed drug concentrations and a wide range of UV exposure levels. This provided a quantitative comparison of the effects of the various acridines and ethidium bromide on DNA repair in this organism. The effects of the different drugs on dark repair were demonstrated equally well in minimal media and in BHI agar. Post-irradiation survival of E. coli B in the absence of drugs plated on minimal or BHI media is illustrated in Figure 6 (Curve A) and Figure 7 (Curve A), respectively. In each instance there was a characteristic decline in viability as a function of the ultraviolet dose.

It was also apparent from these experiments that post-irradiation survival on minimal media was greater than on enriched media.

FIGURE 5

THE DRUG RESPONSE CURVES OF E. COLI ts-7
TO 10-METHYLACRIDINIUM CHLORIDE

Control organisms were plated without UV irradiation. The rest of the organisms received a single UV exposure of 50 ergs/ mm^2. Following irradiation, the organisms were spread on Tryptone agar containing 1.0×10^{-6} to 1.0×10^{-4} M drug. The plates were separated into two groups. One group of plates was incubated in the dark at 40°C for 2 hours, and then placed with the other group which had been incubated in the dark at 25°C. The total incubation for both groups of plates at 25°C was 24-36 hours.

Curve A - organisms grown at 25°C without UV irradiation.

Curve B - organisms grown at 25°C with UV irradiation.

Curve C - organisms grown at 40°C without UV irradiation.

Curve D - organisms grown at 40°C with UV irradiation.

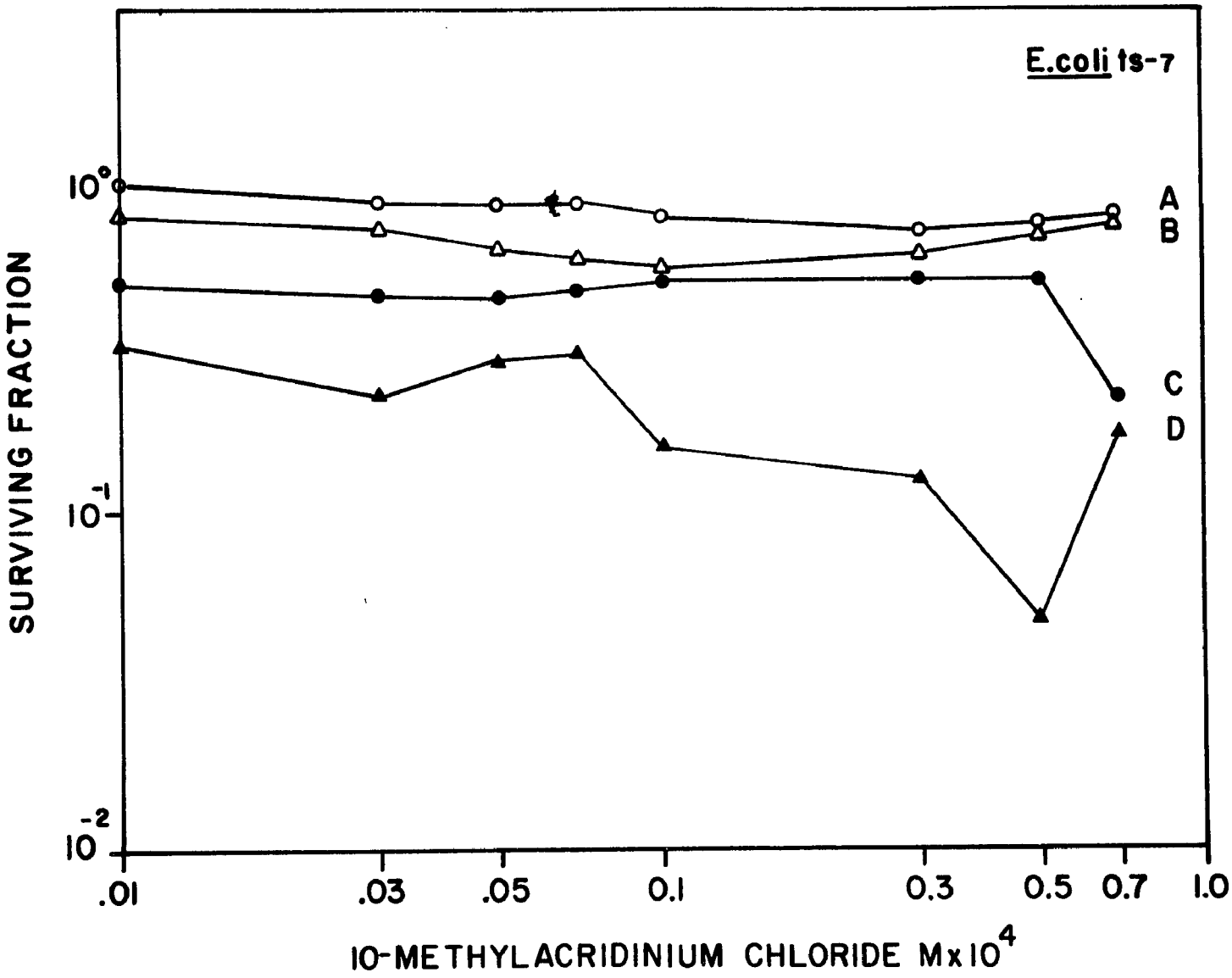

E.coli ts-7
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
A
B
C
D
.01
.03
.05
0.1
0.3
0.5
0.7
1.0
10-METHYLACRIDINIUM CHLORIDE M x 10^{4}

FIGURE 6

THE EFFECT OF THE VARIOUS ACRIDINES AND ETHIDIUM BROMIDE ON THE POST-IRRADIATION SURVIVAL OF *E. COLI* B GROWN ON MINIMAL MEDIA

Following UV irradiation of 0-300 ergs/mm^2, the organisms were spread on media in the presence or absence of the various drugs. The plates were then incubated at 37°C for approximately 36 hours.

Curve A - control, absence of drug

Curve B - 1.0×10^{-6} M 10-methylacridinium chloride

Curve C - 1.0×10^{-6} M acridine orange

Curve D - 1.0×10^{-6} M ethidium bromide

Curve E - 1.0×10^{-6} M 9-aminoacridine hydrochloride

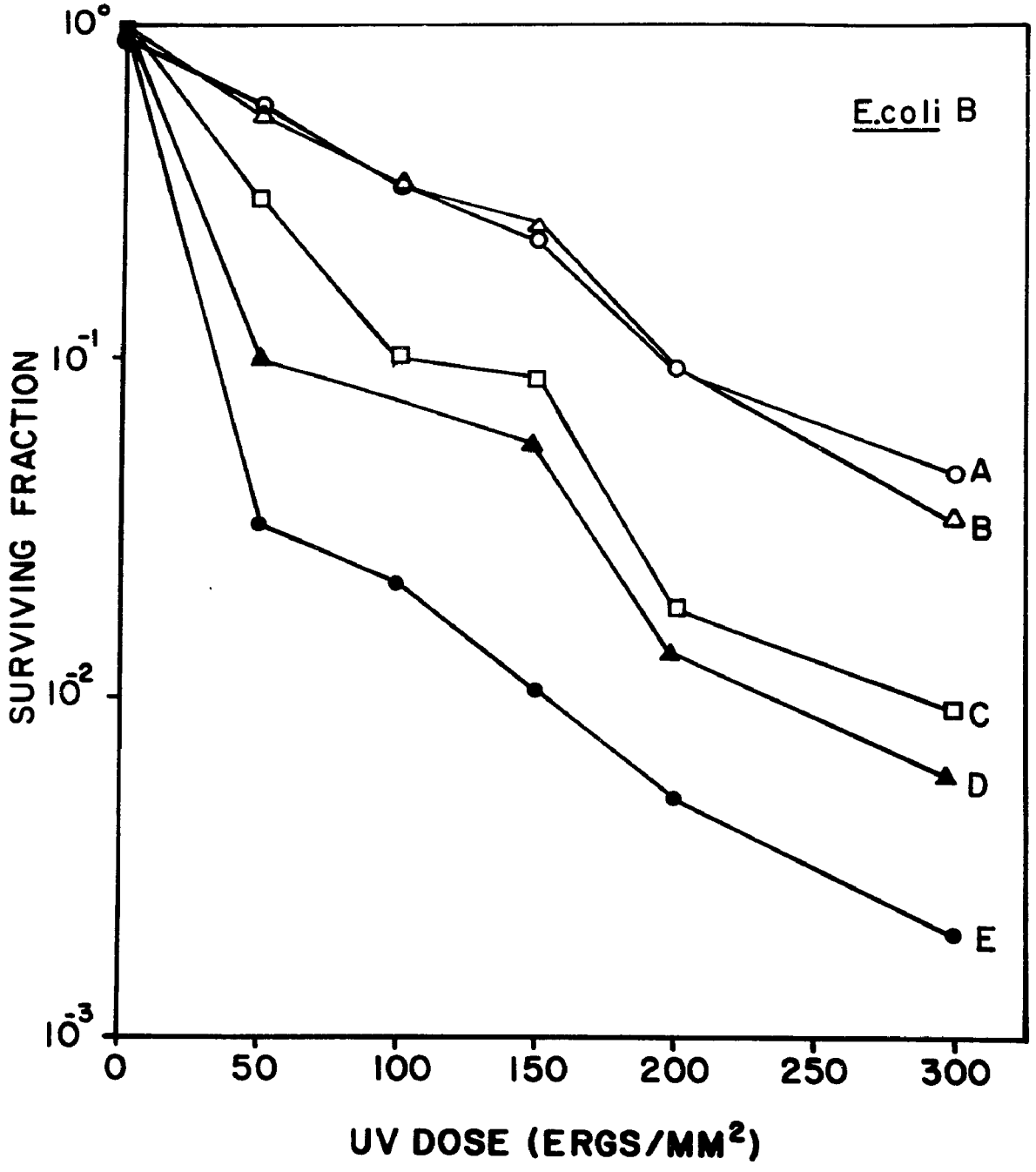

E.coli B
SURVIVING FRACTION
10°
10⁻¹
10⁻²
10⁻³
0
50
100
150
200
250
300
UV DOSE (ERGS/MM²)
A
B
C
D
E

FIGURE 7

THE EFFECT OF THE VARIOUS ACRIDINES AND ETHIDIUM BROMIDE ON THE POST-IRRADIATION SURVIVAL OF E. COLI B GROWN ON BHI AGAR

Following UV irradiation of 0-300 ergs/mm^2, the organisms were spread on media in the presence or absence of the various drugs. The plates were then incubated overnight in the dark at 37°C.

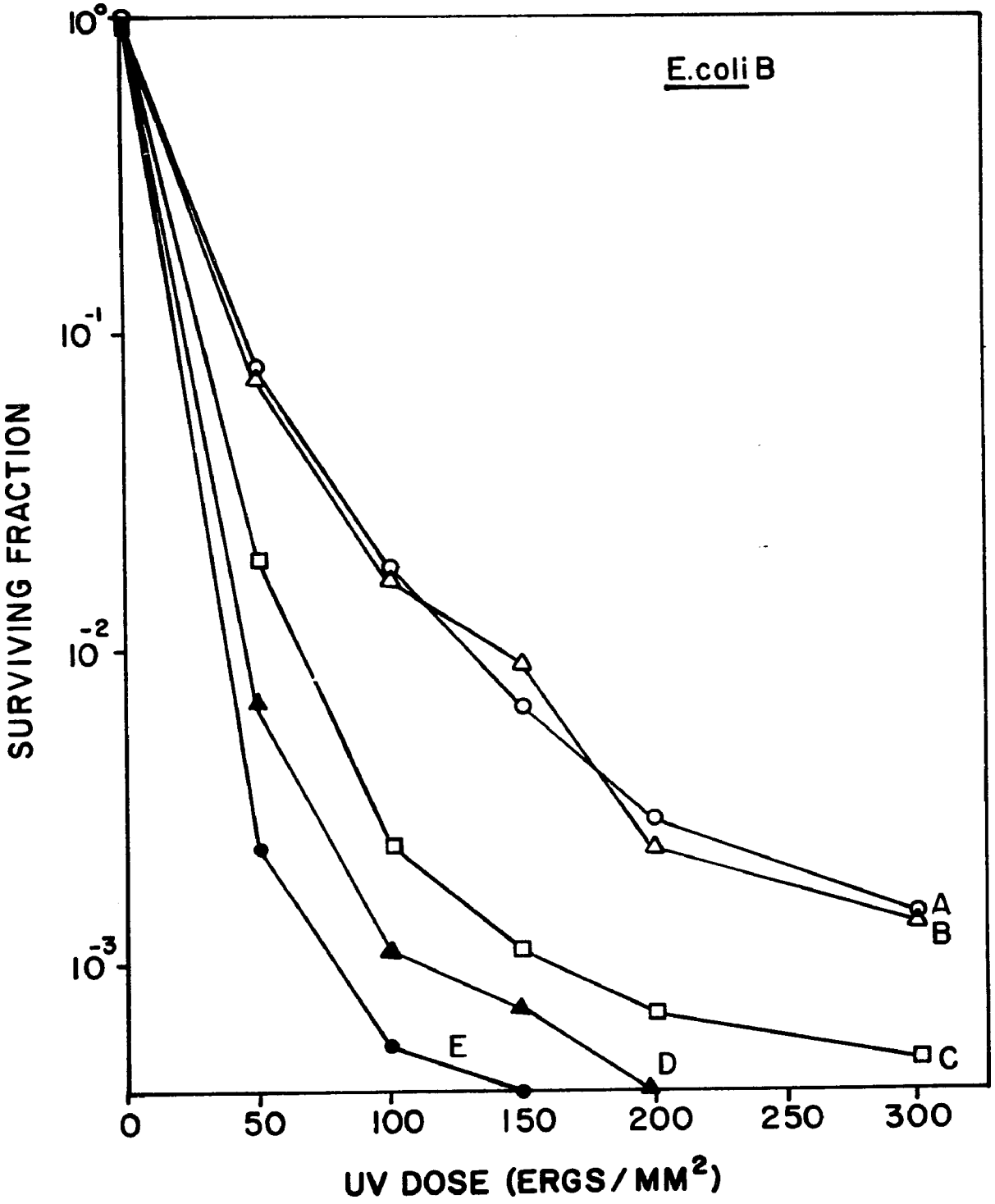
Curve A - control, absence of drug

Curve B - 1.0×10^{-6} M 10-methylacridinium chloride

Curve C - 1.0×10^{-6} M acridine orange

Curve D - 1.0×10^{-6} M ethidium bromide

Curve E - 1.0×10^{-6} M 9-aminoacridine hydrochloride


E.coli B
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
10^{-3}
A
B
C
D
E
0
50
100
150
200
250
300
UV DOSE (ERGS/MM^2)

This observation is explained by the fact that repair and cell division are both time dependent processes. E. coli B growing on minimal media divide every 60 minutes, whereas they divide every 20 minutes on BHI. Thus, organisms growing on minimal media have a longer time between cell divisions, and therefore more time to repair their damaged DNA. In contrast, organisms growing on BHI must divide more often and consequently are killed because of lack of sufficient time for repair.

At zero UV exposure, where the samples have not been irradiated, the survival was not decreased by the acridines or ethidium bromide at 1.0×10^{-6} M. This concentration was well below the optimum mutagenic concentration for acridines reported by Orgel and Brenner (34).

The effects of 9-aminoacridine hydrochloride, acridine orange and 10-methylacridinium chloride on post-irradiation survival varied directly with mutagenicity. The strongest mutagen, 9-aminoacridine hydrochloride caused the largest decrease in survival (Curve E). For example, after a dose of 50 ergs/mm^2, survival on minimal media (Figure 6) containing 9-aminoacridine hydrochloride decreased to 3% as compared to approximately 60% in the absence of this drug. In contrast, the non-mutagen (26), 10-methylacridinium chloride caused no decrease in post irradiation survival (Curve B).

Acridine orange, which is less mutagenic than 9-aminoacridine

hydrochloride was correspondingly less inhibitory toward survival following UV irradiation (Curve C). Ethidium bromide, whose mutagenicity has not been studied in E. coli, showed inhibition of UV survival although it was less inhibitory than 9-aminoacridine hydrochloride. Thus, these experiments demonstrated that mutagenicity appeared to be correlated with inhibition of the dark repair of UV damage in E. coli B.

E. coli B_{s-1}

These preliminary findings with E. coli B indicated that dark repair was the process being inhibited by the drugs. In order to assure that the mode of action was directed specifically toward DNA excision repair rather than simply inhibiting the normal DNA replication of UV damaged organisms, E. coli B_{s-1}, a mutant unable to perform the first step of DNA dark repair, but possessing normal replicative enzymes and complete photoreactivation (39) and post-replicative repair mechanisms (38) was chosen for study. The use of this mutant could also rule out alternate repair mechanisms.

For the post-irradiation study, this organism was grown with and without 1.0×10^{-6} M 9-aminoacridine hydrochloride in BHI agar. In Figure 8, Curve A represents the survival of organisms without the drug, and Curve B shows the survival in the presence of the drug. These data show that 1.0×10^{-6} M 9-aminoacridine hydrochloride

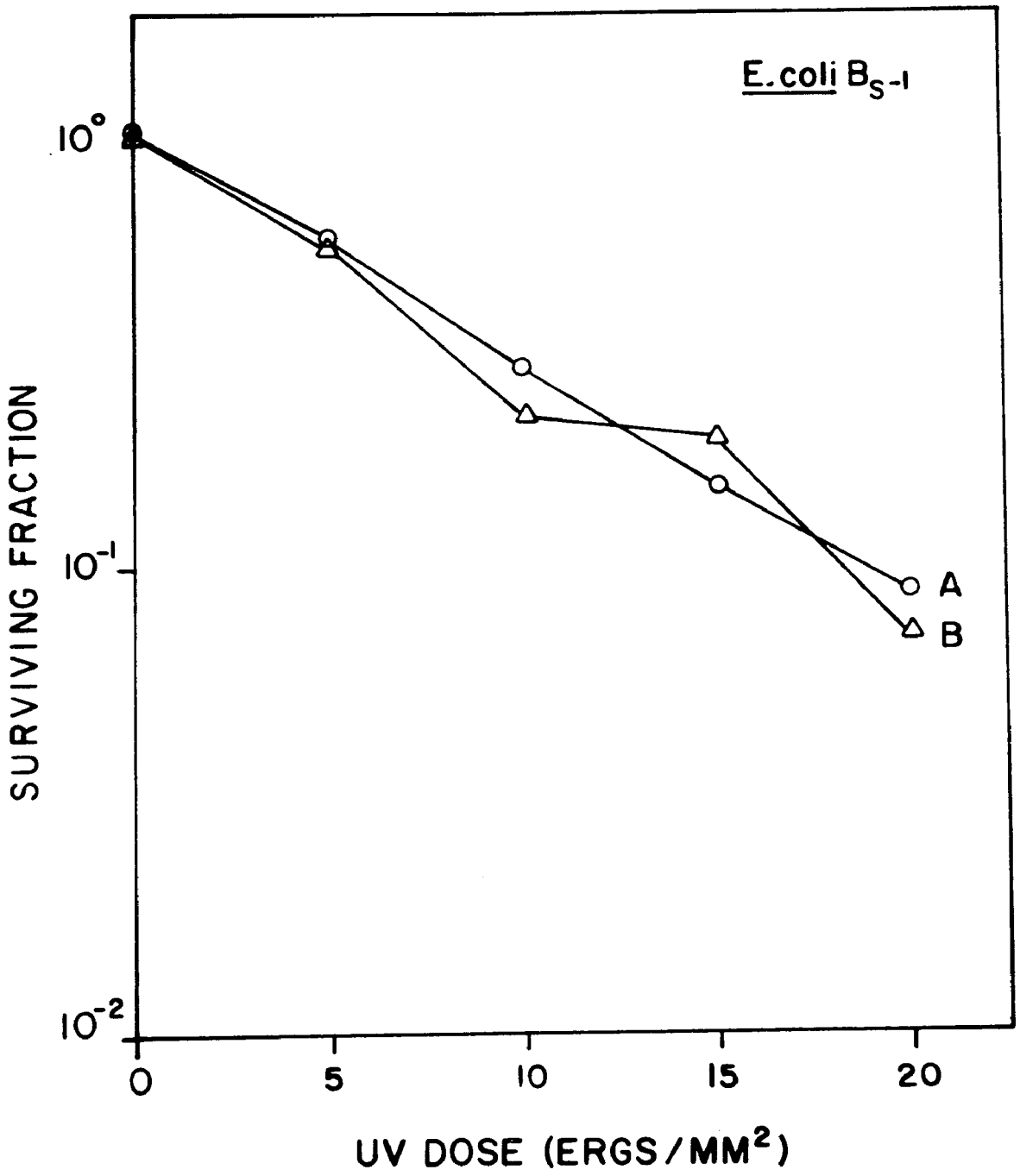
FIGURE 8

THE EFFECT OF 9-AMINOACRIDINE HYDROCHLORIDE
ON THE POST-IRRADIATION SURVIVAL OF *E. COLI* B_s-1

Following UV irradiation of 0-20 ergs/mm^2, the organisms were spread on BHI agar and incubated overnight in the dark at 37°C.

Curve A - control, absence of drug

Curve B - 1.0×10^{-6} M 9-aminoacridine hydrochloride


E. coli B_{S-1}
10^{0}
10^{-1}
10^{-2}
SURVIVING FRACTION
0
5
10
15
20
UV DOSE (ERGS/MM^2)
A
B

has no effect on post-irradiation survival of E. coli B_{s-1}. As expected, the sensitivity of this organism to UV light was increased sharply over that of E. coli B, and a relatively low dose (20 ergs/mm^2) killed approximately 90% of the organisms.

Since this organism was not able to perform the incision step, it could not initiate the dark repair process. The finding that 9-aminoacridine hydrochloride was unable to inhibit the post-irradiation survival of E. coli B_{s-1} supported the hypothesis that the actual process being inhibited by the drug in E. coli B was dark repair.

E. coli B/r

E. coli B/r is a mutant which is highly resistant to irradiation and capable of surviving 10 times the lethal dose of radiation for E. coli B (22). The difference between the resistance in E. coli B and E. coli B/r seems to be related to the observation that after UV irradiation, E. coli B begins to form filaments, and E. coli B/r does not (49). However, the correlation between UV irradiation and the initiation of filament formation is not understood. The ability to repair high levels of UV damage makes this organism interesting for comparison with E. coli B, because this is E. coli B/r's single mutant property.

The post-ultraviolet survival of E. coli B/r, grown with and

without 1.0×10^{-6} M 9-aminoacridine hydrochloride, is shown in Figure 9. The survival of control organisms found in Curve A reflects the ability of these organisms to survive large doses of UV irradiation. Curve B shows the decrease in survival of organisms plated with the drug. As can be seen, at a dose of 400 ergs/mm^2, survival of control organisms had decreased to 50 percent, but survival of organisms growing on media containing 1.0×10^{-6} M 9-aminoacridine hydrochloride was decreased to approximately 20 percent.

Although this organism is highly resistant to irradiation, massive doses of UV light produced a characteristic decline in viability. Moreover, the drug decreased post-irradiation survival as in the case of E. coli B.

E. coli ts-7

The evidence from these experiments strongly implicates dark repair as the process being inhibited by acridines. Since DNA repair is a multi-step enzymatic process, it is conceivable that the drug might be exerting its effect on one or more of the individual enzyme steps, i.e. incision, excision, polymerization, or rejoining. Inhibition of a particular step in the dark repair process can be studied in vivo using microorganisms containing single point mutations for the different steps of repair. Such a mutation has been reported for E. coli ts-7 (12,35).

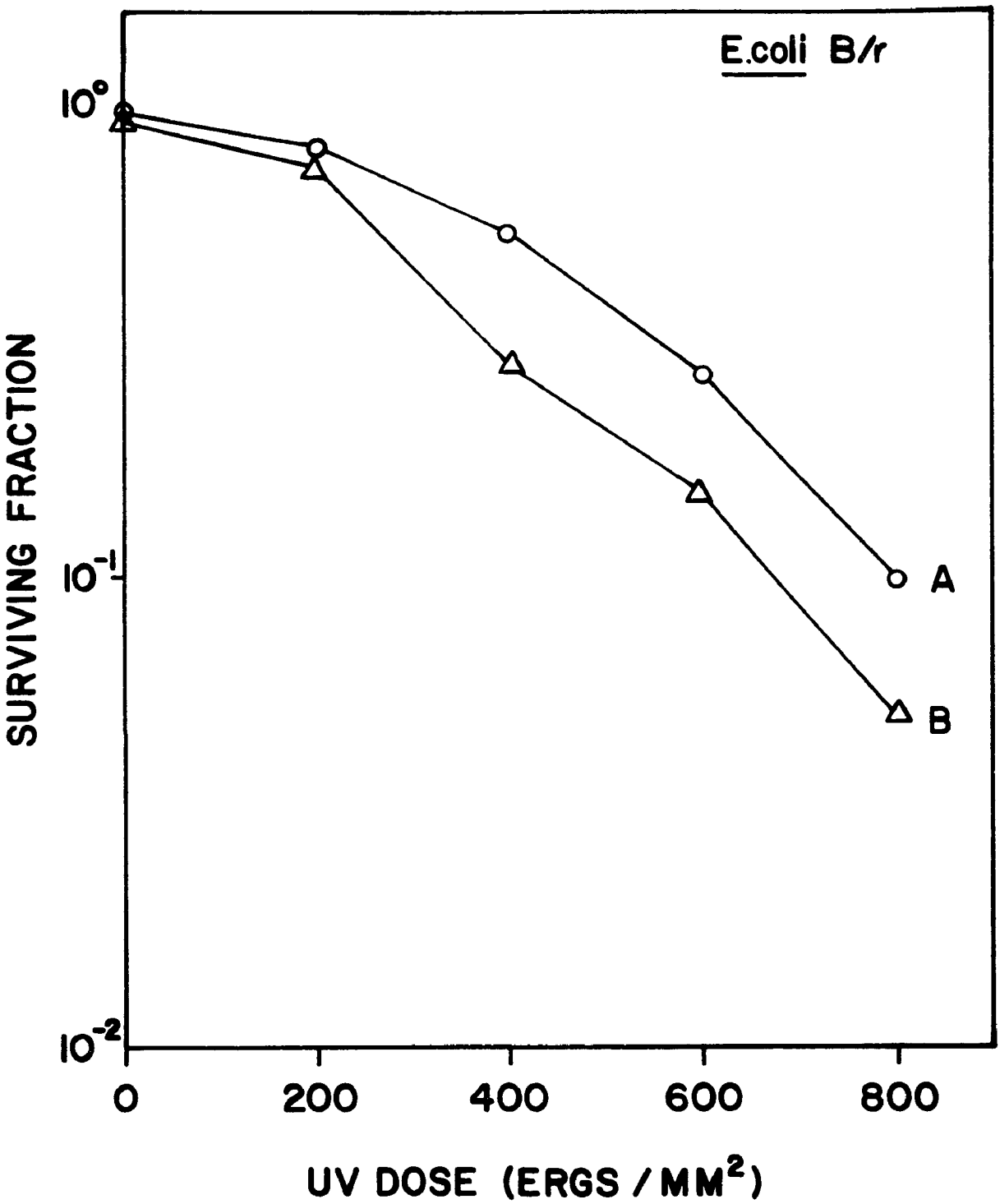
FIGURE 9

THE EFFECT OF 9-AMINOACRIDINE HYDROCHLORIDE
ON THE POST-IRRADIATION SURVIVAL OF E. COLI B/r

Following UV irradiation of 0-800 ergs/mm^2, the organisms were spread on BHI agar and then incubated overnight in the dark at 37°C.

Curve A - control, absence of drug

Curve B - 1.0×10^{-6} M 9-aminiacridine hydrochloride


E.coli B/r
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
A
B
0
200
400
600
800
UV DOSE (ERGS / MM^2)

This mutant, E. coli ts-7, was derived from E. coli TAU-bar using the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (35). Pauling and Hamm studied the properties of this mutant by examining 1) its ability to excise thymine dimers, 2) its rate of repair synthesis, and 3) its sensitivity to thymineless death. They concluded that the organism was a temperature sensitive ligase mutant. The organism exhibited normal ligase properties at 25°C, but showed marked decrease in survival when incubated 2 hours at 40°C following UV irradiation. In comparison, incubation at 40°C had no effect on unirradiated organisms (35).

The post-irradiation survival of E. coli ts-7 spread on plates with and without 1.0×10^{-5} M 9-aminoacridine hydrochloride is shown in Figure 10. The survival of control organisms grown at 25°C and 40°C is shown by Curves A and C. Curves B and D reflect a decreased post-irradiation survival at 25°C and 40°C of organisms plated on the acridine.

As stated previously, at 40°C this temperature sensitive ligase mutant shows decreased enzyme activity either by inactivation of the protein or by decreased synthesis. This temperature sensitivity is most likely due to a few amino acid substitutions which render the enzyme more susceptible to heat denaturation. The denatured enzyme 1) might not bind substrate, cofactors or regulators normally, or 2) might not be able to catalyze the reaction correctly or

FIGURE 10

THE EFFECT OF 1.0×10^{-5} M 9-AMINOACRIDINE HYDROCHLORIDE ON THE POST-IRRADIATION SURVIVAL OF E. COLI ts-7

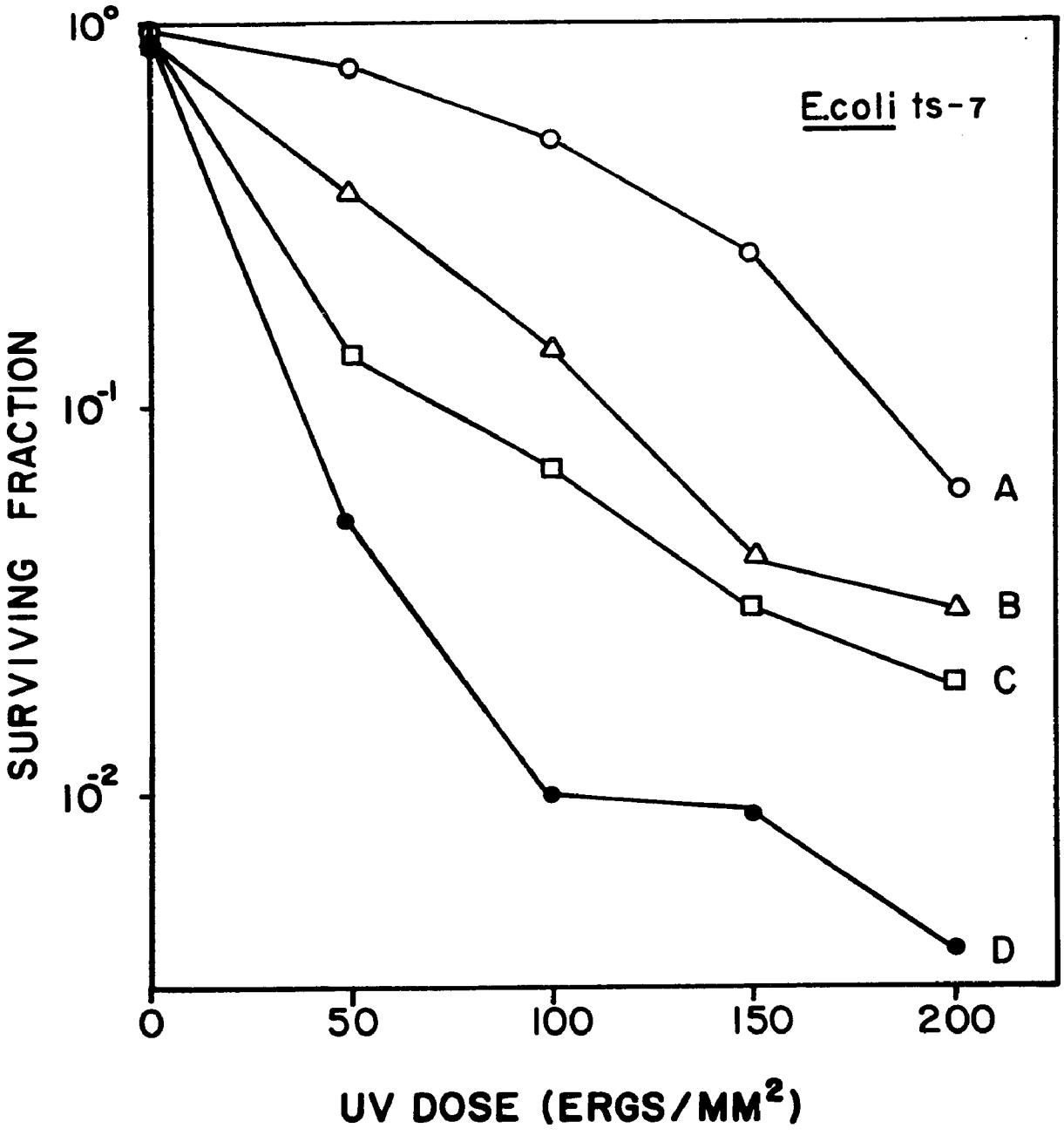
Following UV irradiation of 0-200 ergs/mm^2, the organisms were spread on Tryptone agar in the presence and absence of the drug. The plates were separated into two groups. One group of plates was incubated in the dark at 40°C for 2 hours, and then placed with the other group which had been incubated in the dark at 25°C. The total incubation for both groups of plates at 25°C was 24-36 hours.

Curve A - organisms grown at 25°C in the absence of drug.

Curve B - organisms grown at 25°C with 1.0×10^{-5} M 9-aminoacridine hydrochloride.

Curve C - organisms grown at 40°C in the absence of drug.

Curve D - organisms grown at 40°C with 1.0×10^{-5} M 9-aminoacridine hydrochloride.


E.coli ts-7
SURVIVING FRACTION
10°
10⁻¹
10⁻²
A
B
C
D
0
50
100
150
200
UV DOSE (ERGS/MM²)

completely. This enzyme performs the terminal step in repair, and it can only repair those sites which have been acted on completely during the preceeding steps of repair. Since the drug at 40°C did not eliminate survival but produced an inhibition of post-irradiation survival comparable to that observed at 25°C, the effect of the drug on dark repair did not appear to be at the ligase step. This finding indicates that the drug may either act exclusively on some other enzymic step in the repair process or inhibit the overall repair process.

Figure 11 shows the post-irradiation survival of E. coli ts-7 plated on Tryptone agar with and without 1.0×10^{-6} M 9-aminoacridine hydrochloride. Curves A and C represent post-irradiation survival of the controls at 25°C and 40°C, respectively. The survival of these organisms in the presence of the drug is depicted by Curves B and D. Organisms which were plated on media containing 1.0×10^{-6} M 9-aminoacridine hydrochloride displayed no decrease in survival when compared to the controls at either 25°C or 40°C. Also, post-irradiation survival of treated and untreated E. coli ts-7 is greater at 25°C than at 40°C.

Comparing post-irradiation survival of E. coli ts-7 at 1.0×10^{-6} M and 1.0×10^{-5} M 9-aminoacridine hydrochloride, no decrease in survival was observed at 1.0×10^{-6} M, but marked decline of survival was noted at 1.0×10^{-5} M. In contrast, 1.0×10^{-6} M

FIGURE 11

THE EFFECT OF 1.0×10^{-6} M 9-AMINOACRIDINE HYDROCHLORIDE ON THE POST-IRRADIATION SURVIVAL OF *E. COLI* ts-7

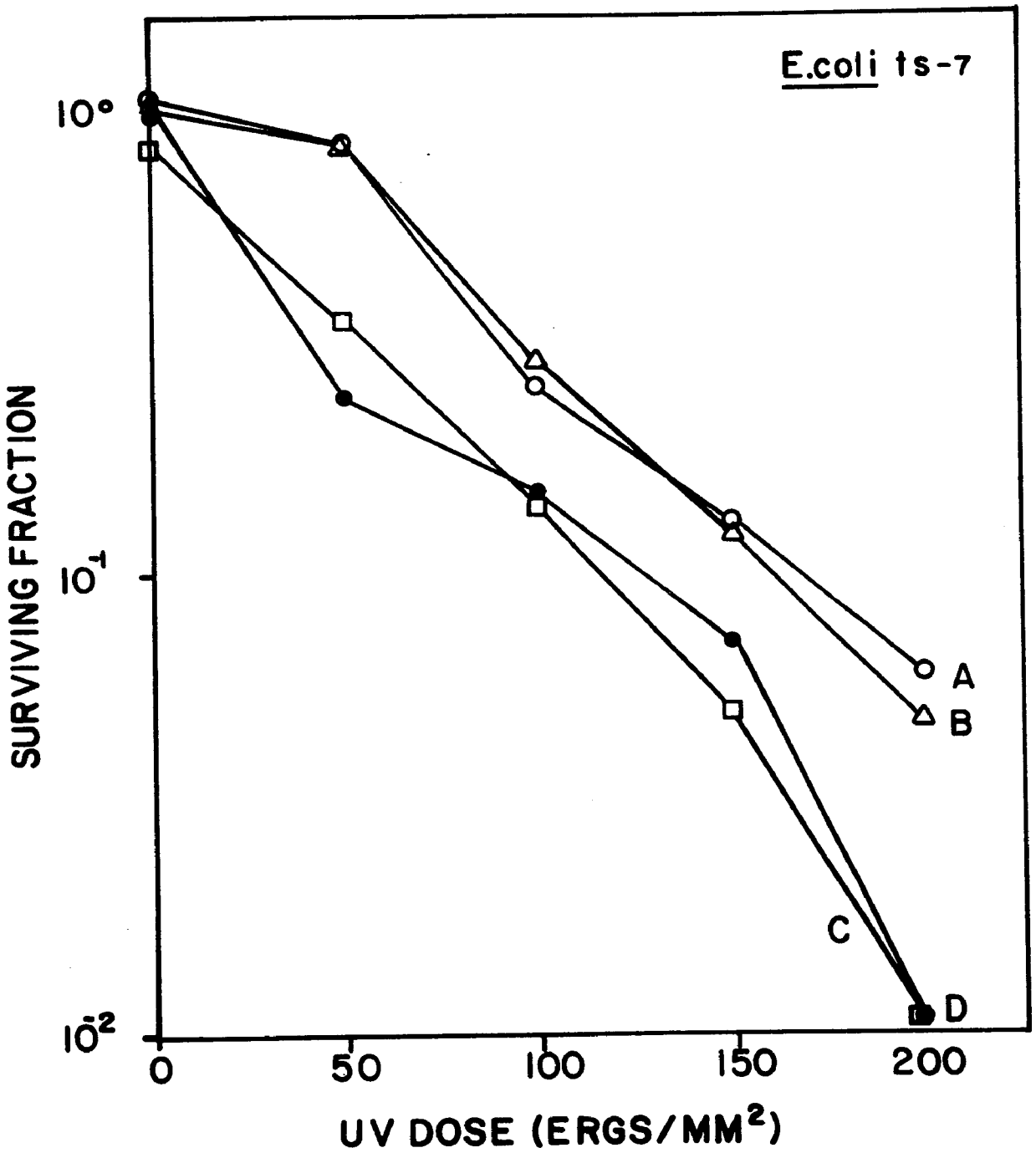
Following UV irradiation of 0-200 ergs/mm^2, the organisms were spread on Tryptone agar in the presence and absence of drug. The plates were separated into two groups. One group of plates was incubated in the dark at 40°C for 2 hours, and then placed with the other group which had been incubated in the dark at 25°C. The total incubation for both groups of plates at 25°C was 24-36 hours.

Curve A - organisms grown at 25°C in the absence of drug.

Curve B - organisms grown at 25°C with 1.0×10^{-6} M 9-aminoacridine hydrochloride.

Curve C - organisms grown at 40°C in the absence of drug.

Curve D - organisms grown at 40°C with 1.0×10^{-6} M 9-aminoacridine hydrochloride.


E.coli ts-7
SURVIVING FRACTION
10°
10⁻¹
10⁻²
0
50
100
150
200
UV DOSE (ERGS/MM²)
A
B
C
D

9-aminoacridine hydrochloride was shown to be a strong inhibitor of DNA repair in E. coli B. These differences in the effective concentration of the drug are unexplained but may possibly be due to some property unrelated to the dark repair. Accordingly, it became highly desirable to do experiments with the parent strain of this organism.

E. coli TAU-bar

E. coli TAU-bar is the parent strain of E. coli ts-7 and served as an ideal control. It grows normally at 25°C exhibiting normal ligase activity. In contrast to E. coli ts-7 this organism shows enhanced UV survival at 40°C. The post-irradiation survival response of the control organism E. coli TAU-bar at 25°C is shown in Figure 12. Curve A represents the survival of control organisms plated on media in the absence of the drug, and Curve B reflects the survival of organisms plated on media containing 1.0×10^{-6} M 9-aminoacridine hydrochloride. These organisms behaved similarly to E. coli ts-7 and displayed no decrease in survival when exposed to the drug at this concentration.

The post-irradiation survival of E. coli TAU-bar incubated for 2 hours at 40°C is shown in Figure 13. Curve A represents survival of the untreated controls, while Curve B is survival of the organisms plated on 1.0×10^{-6} M 9-aminoacridine hydrochloride after

FIGURE 12

THE EFFECT OF 1.0×10^{-6} M 9-AMINOACRIDINE HYDROCHLORIDE ON THE POST-IRRADIATION SURVIVAL OF E. COLI TAU-bar AT 25°C

Following UV irradiation of 0-200 ergs/mm^2, the organisms were spread on Tryptone agar in the presence and absence of the drug. The plates were then incubated in the dark at 25°C for approximately 36 hours.

Curve A - organisms grown at 25°C in the absence of drug.

Curve B - organisms grown at 25°C with 1.0×10^{-6} M 9-aminoacridine hydrochloride.

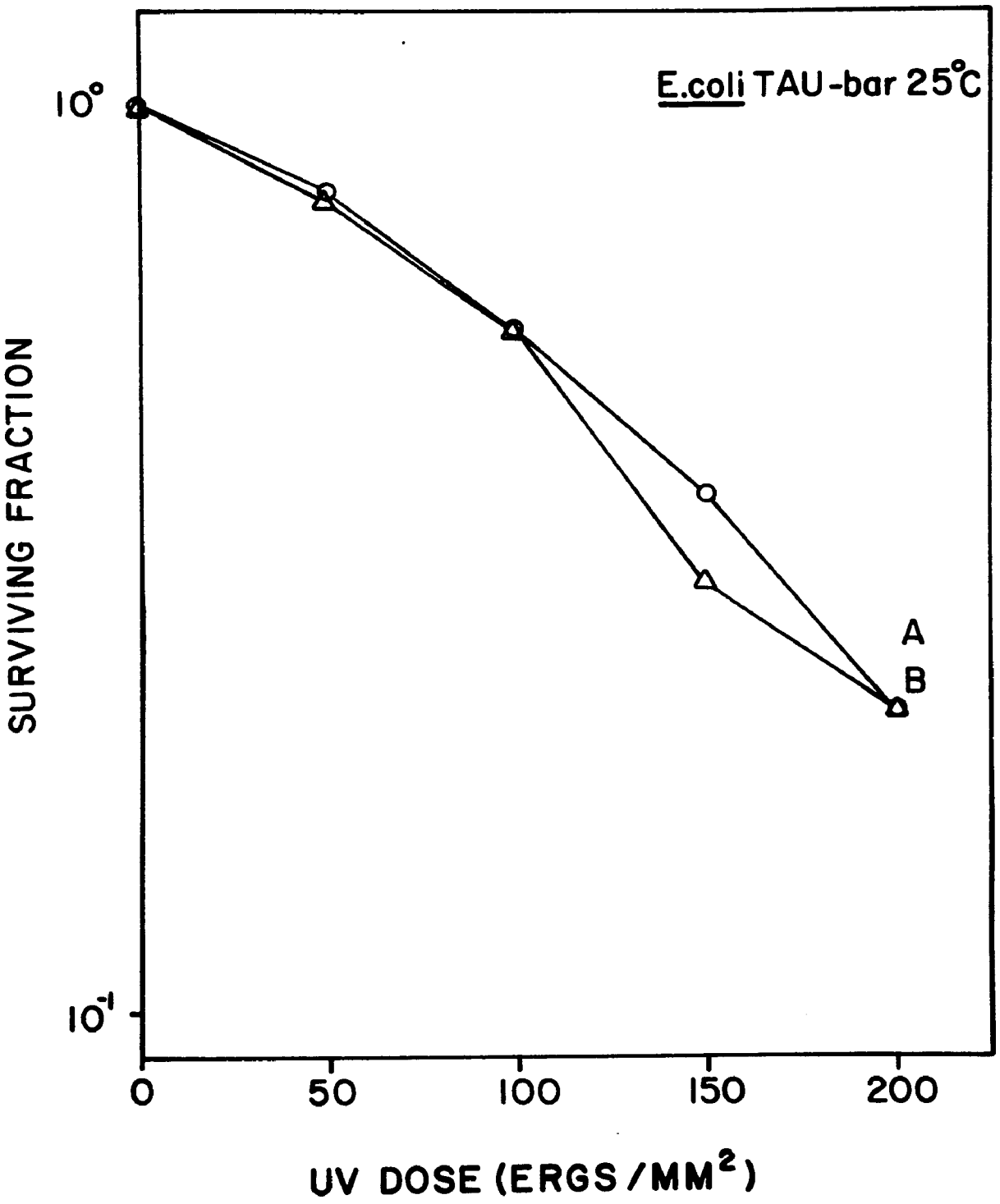

E.coli TAU-bar 25°C
10°
10⁻¹
SURVIVING FRACTION
A
B
0
50
100
150
200
UV DOSE (ERGS/MM²)

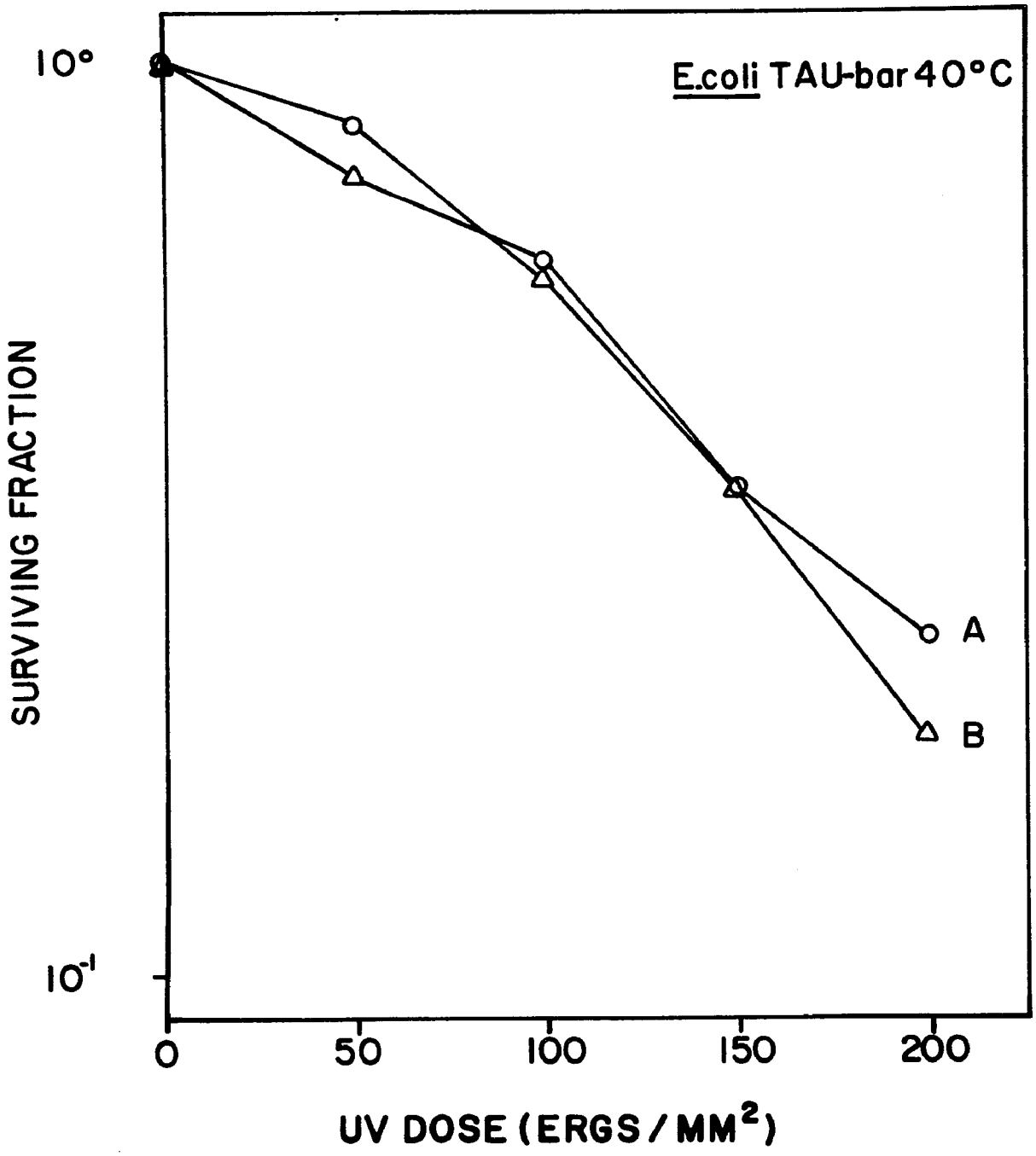
FIGURE 13

THE EFFECT OF 1.0 x 10^{-6} M 9-AMINOACRIDINE HYDROCHLORIDE ON THE POST-IRRADIATION SURVIVAL OF E. COLI TAU-bar AT 40°C

Following UV irradiation of 0-200 ergs/mm^2, the organisms were spread on Tryptone agar in the presence and absence of drug. The plates were then incubated in the dark at 40°C for 2 hours. Subsequently, the plates were incubated in the dark at 25°C for about 24 hours.

Curve A - organisms grown at 40°C in the absence of drug.

Curve B - organisms grown at 40°C with 1.0 x 10^{-6} M 9-aminoacridine hydrochloride.


E.coli TAU-bar 40°C
10°
10⁻¹
SURVIVING FRACTION
A
B
0
50
100
150
200
UV DOSE (ERGS / MM²)

irradiation. Curves A and B show the noneffectiveness of the drug to inhibit dark repair at this concentration.

The survival of E. coli TAU-bar with and without 1.0×10^{-5} M 9-aminoacridine hydrochloride is shown in Figure 14. In the absence of drug, E. coli TAU-bar shows greater survival at 40°C than at 25°C. These findings agree with those of Pauling and Hamm (35). Curves A and C represent the UV survival of organisms grown in the absence of drug at 40°C and 25°C, respectively. Curves B and D show inhibition of dark repair of UV damage by 1.0×10^{-5} M 9-aminoacridine hydrochloride at 40°C and 25°C, respectively. As found with E. coli ts-7, 1.0×10^{-5} M drug was required for inhibition of repair although survival was not decreased as drastically as with E. coli B.

E. coli TAU-bar and E. coli ts-7 are both poly-auxotrophic mutants, i.e., they require the addition of uracil, thymine, methionine, proline, arginine and tryptophan for growth on minimal media. Their generation time on supplemented minimal media is approximately 2.5 hours, and on Tryptone media approximately one hour. This prolonged generation time confers a selective advantage on the organism when it is affronted with ultraviolet radiation damage, because it allows the organism more time to repair DNA.

The decreased drug sensitivity to acridine inhibition of repair is unexplained. However, one factor which may contribute to the

FIGURE 14

THE EFFECT OF 1.0×10^{-5} M 9-AMINOACRIDINE HYDROCHLORIDE ON THE POST-IRRADIATION SURVIVAL OF *E. COLI* TAU-bar

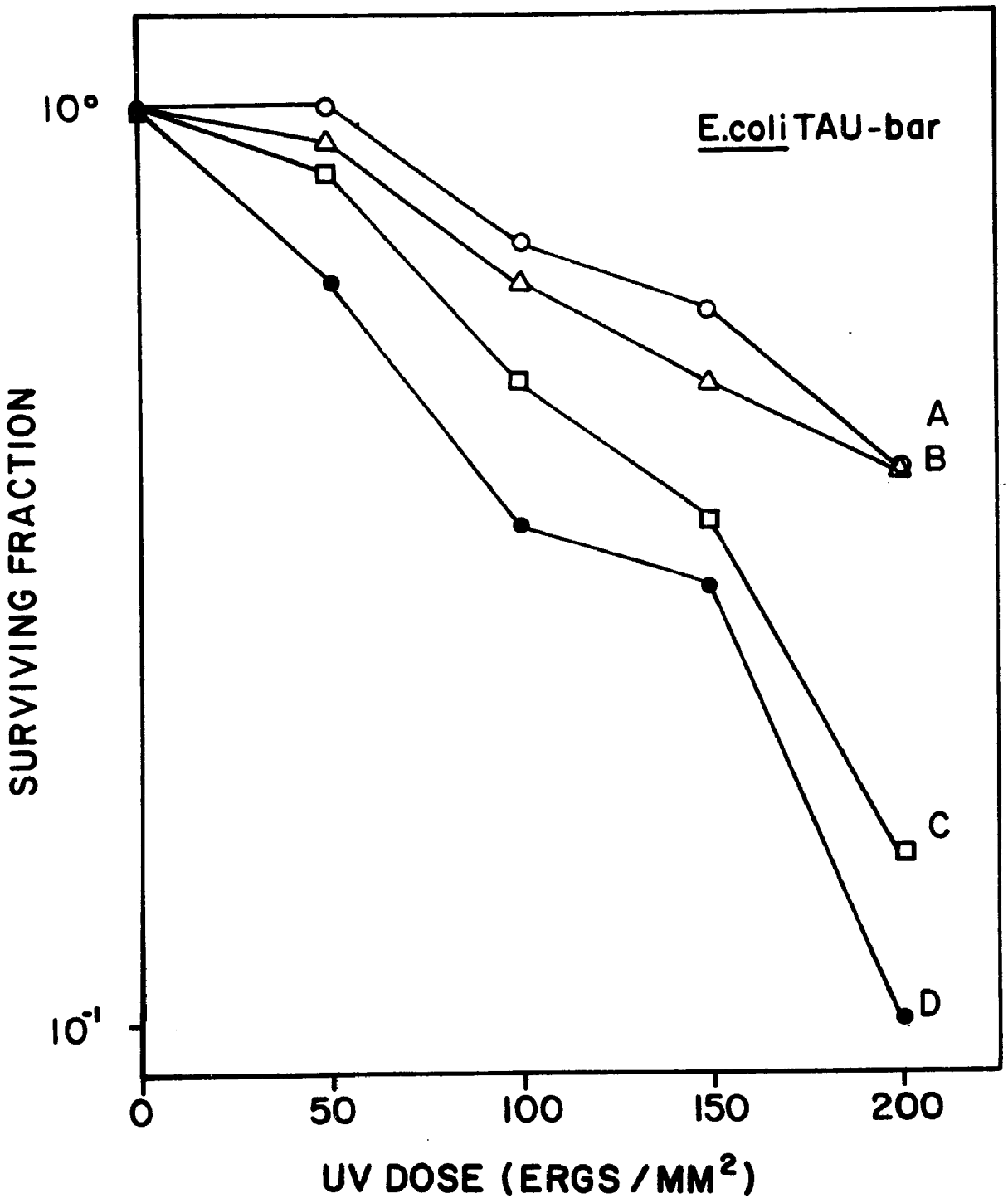
Following UV irradiation of 0-200 ergs/mm^2, the organisms were spread on Tryptone agar in the presence and absence of drug. The plates were separated into two groups. One group of plates was incubated in the dark at 40°C for 2 hours, and then placed with the other group which had been incubated in the dark at 25°C. The total incubation for both groups of plates at 25°C was 24-36 hours.

Curve A - organisms grown at 40°C in the absence of drug.

Curve B - organisms grown at 40°C with 1.0×10^{-5} M 9-aminoacridine hydrochloride.

Curve C - organisms grown at 25°C in the absence of drug.

Curve D - organisms grown at 25°C with 1.0×10^{-5} M 9-aminoacridine hydrochloride.


E.coli TAU-bar
10°
10⁻¹
SURVIVING FRACTION
0
50
100
150
200
UV DOSE (ERGS / MM²)
A
B
C
D

decreased drug sensitivity in E. coli TAU-bar and E. coli ts-7 may be their inherently longer generation time. In the case of E. coli B grown on minimal media, where the generation time is approximately 60 minutes, a UV dose of 50 ergs/mm^2 decreases survival to 60%. With E. coli B grown on BHI agar and having a generation time of 20 minutes, a UV dose of 50 ergs/mm^2 reduces survival to about 10%. The generation time for organisms grown on BHI media is reduced, because this nutrient medium provides all the essential pre-synthesized constituents for cellular growth. In minimal media, however, the organisms must convert glucose, the sole carbon source, into the compounds required for cellular proliferation. In either media, as soon as a predetermined level of the cellular constituents is reached, cell division occurs, regardless of the state of DNA repair. Thus, in some instances, cell division is lethal, because DNA repair of UV damage is incomplete.

Thus, generation time may be a factor which contributes to the organism's ability to survive UV irradiation. In the case of E. coli B, post-irradiation survival was greatly enhanced when these organisms were allowed a longer time to repair their UV damaged DNA when grown on minimal media. Because E. coli TAU-bar and E. coli ts-7 have inherently longer generation times even when grown on nutrient media, the extent of killing by UV irradiation is less than that for E. coli B.

Other, perhaps more important, factors bestowing enhanced post-irradiation survival on E. coli TAU-bar and E. coli ts-7 may be 1) an altered rate-limiting step in the repair process, or 2) modified cell wall permeability. A shift in the rate-limiting step of repair, which acts to accelerate the overall repair process, may result inherently through mutation. This is a likely possibility, because this organism's genome has undergone extensive chemically induced alterations. Also, the presence of an excess amount of chemical nutrients may act to increase the efficiency of some of the synthetic processes and thus, conceivably affect the rate-limiting step in excision repair. Again, because of the number of mutagenic events this organism has undergone, the possibility that the cell membrane has been altered cannot be ruled out. The investigation of cell wall permeability to various mutagens, which has not as yet been undertaken, might provide insight into the drug effect on the processes of dark repair and mutagenesis.

IV. DISCUSSION

E. coli possesses the wild-type genome which is the natural unmutated form of the gene. Mutations, either spontaneous or induced, may be expressed by altered growth requirements or changed physical characteristics. The ultimate product of non-lethal mutations is a new organism, for example, E. coli B_s-1 which was a spontaneous mutant of E. coli B.

In order to achieve a wide spectrum of survival responses reflecting the ability to repair UV damage, E. coli B and two repair mutants were chosen for study: 1) E. coli B because of its normal survival response to UV irradiation, 2) E. coli B_s-1, because of its drastically decreased survival after UV irradiation (21), and 3) E. coli B/r, because of its enhanced survival response to UV damage (22).

E. coli B has a generation time of about 20 minutes on nutrient media and 60 minutes on minimal media (49). It is a non-auxotrophic organism capable of growing with glucose as its sole carbon source, because it synthesizes all of its essential chemical components from this simple sugar and necessary salts. This organism repairs UV damage to its DNA using all three of the known modes of repair, viz. photoreactivation, dark repair and recombinational repair (53).

Also, its ability to repair UV damage is enhanced on minimal media because the longer generation time offers more time for repair.

E. coli B_{s-1} and E. coli B/r are able to grow on minimal media utilizing glucose as the sole carbon source, and have generation times identical to that of E. coli B (19). E. coli B_{s-1}, however, is a spontaneous mutant which has lost the ability to carry out the incision reaction of dark repair (52). This organism was useful in the drug studies as a control, because it permitted testing of the hypothesis that acridines inhibit post irradiation survival by inhibiting excision repair.

The high resistance to UV irradiation of E. coli B/r made it useful for study, because it performed dark repair more efficiently than E. coli B. The findings of Setlow and Carrier (41), which demonstrated that both organisms release H^3-thymine dimers at an equivalent rate, indicated that E. coli B and E. coli B/r performed incision and excision to the same degree. However, this does not show the extent of polymerization and ligation. In fact, E. coli B/r may have enhanced polymerase and/or ligase activity as reflected by its enhanced survival after massive doses of UV irradiation. It has been reported that E. coli B begins to form external filaments (22,49) after UV irradiation and E. coli B/r does not, and this suggests that the lack of filament formation in E. coli B/r is related to its increased resistance to UV radiation. Filament

formation in E. coli B may utilize energy and compounds needed to complete repair.

E. coli TAU-bar is a polyauxotrophic mutant requiring the addition of arginine, methionine, proline, tryptophan, uracil and thymine for growth on minimal media (35). E. coli ts-7 is a mutant resulting when E. coli TAU-bar is exposed to N-methyl-N'-nitro-N-nitrosoguanidine (5). E. coli ts-7 has the same mutant growth requirements as E. coli TAU-bar. Both organisms have characteristic survival at 25°C and 40°C, but the optimum temperature for growth is 40°C for E. coli TAU-bar and 25°C for E. coli ts-7 (35). When these organisms are grown at 25°C in Tryptone broth, the generation time for E. coli TAU-bar is about 50 minutes, while that for E. coli ts-7 is 60 minutes (35).

E. coli ts-7 is a temperature-sensitive mutant, and following UV irradiation, a 2-hour incubation at 40°C depletes the organism of ligase, either by inactivating it or by preventing normal enzyme synthesis. In summary, the effect of mutagenic and non-mutagenic acridines on dark repair in E. coli could be examined using E. coli B to establish the drug effect and then E. coli ts-7, a temperature-sensitive mutant, to determine if acridine inhibition was at the ligase step.

The strongest inhibitor of post-irradiation survival in E. coli B was 9-aminoacridine hydrochloride. This compound was the strongest

mutagen tested, and the concentration needed for inhibition of dark repair is only 1/40 of the concentration required for optimum mutagenesis.

Ethidium bromide has been shown to be mutagenic in yeast (44), and its mode of interaction with DNA is also by intercalation (4,47). Although ethidium bromide does not have a quinacrine ring system, it does possess some similarities to the acridines. Both compounds contain a charged aromatic ring nitrogen and a conjugated ring system. Thus, both ethidium bromide and acridines may participate in charge-charge or ring stacking interactions. Because of its chemical similarities to acridines, and more importantly because of its known mechanism of interaction with DNA, i.e. intercalation, ethidium bromide was also an ideal model compound for these studies.

These investigations provided evidence that ethidium bromide was comparable to 9-aminoacridine hydrochloride in inhibiting post-irradiation survival of E. coli B. This suggests that the acridines may act similarly to ethidium bromide, and that the intercalation mode of binding is relevant to repair inhibition.

Acridine orange, a moderate mutagen, was likewise a moderate inhibitor of DNA repair. The optimum concentration for repair inhibition was 1/10 of the concentration required for mutagenicity. As was the case with 9-aminoacridine hydrochloride, there appears to be a correlation between mutagenicity and ability to inhibit dark

repair, and the acridines appear to be more efficient dark repair inhibitors than mutagens.

10-Methylacridinium chloride, which is non-mutagenic, was also non-inhibitory to DNA repair. However, Riva (37) demonstrated that both mutagenic and non-mutagenic acridines interact with DNA by the same mechanism. The discovery that two compounds with identical binding properties but opposite effects on inhibiting DNA repair demonstrates that the ability to inhibit dark repair cannot be ascertained by determining the mechanism of interaction. Thus, even though ethidium bromide and 9-aminoacridine hydrochloride interact with DNA by intercalation, their mechanism for mutagenesis may involve additional properties.

When Lerman (26) reported that 10-methylacridium chloride was non-mutagenic, he failed to furnish experimental details and neglected mentioning whether this compound is able to enter the cell. However, it seems reasonable to assume that the cell wall would be similarly permeable to mutagenic and non-mutagenic acridines, because the methyl group is the only substitution that renders the mutagenic acridine non-mutagenic.

The effectiveness of mutagenic and non-mutagenic acridines now appears to be related to their chemical structure. The methyl substitution on the ring nitrogen in the non-mutagen (10-methylacridinium chloride) is the only structural characteristic present on

the non-mutagens which is absent on the mutagens. Also, in comparing the relative mutagenicity, 9-aminoacridine hydrochloride, the strong mutagen, has a 9-amino substitution which is para to the ring nitrogen. This para amino group is very electron donating and stabilizes the nitrogen's positive charge over the ring system.

Thus, it appears that on the acridine, the mutagenic and repair inhibitory site is separate from the DNA binding site, and probably involves the quinacrine ring nitrogen. If the nitrogen is methylated, it is blocked and inaccessible, and the acridine is rendered non-inhibitory. A para substitution with electron donating groups increases the reactivity of the nitrogen ring enhancing the inhibitory ability of the drug. Structural differences in the acridines may affect their permeation into the cell. The ability to inhibit dark repair or cause mutations is directly related to the drug's capacity to get into the cell. A methyl substitution on the quinacrine ring nitrogen may render the acridine non-mutagenic by converting its structure into one which cannot permeate the cell wall. The para substitution on the quinacrine ring may provide a structure with an enhanced ability to penetrate into the cell.

Assuming a differential in permeability does not explain how the acridines vary in activity, two models for acridine inhibition of DNA repair are proposed. In the first model, the drug first

binds to DNA exposing the acridine ring nitrogen in its dark repair inhibitory site. The nitrogen may act to attract repair enzymes and form a DNA-acridine-enzyme complex which inactivates the repair enzyme. Alternatively, the nitrogen may repel the enzyme, thereby preventing the formation of a DNA-enzyme complex resulting in loss of repair function. If the nitrogen in the inhibitory site is methylated, the nitrogen is masked, and it exerts no effect on the repair enzyme. If the nitrogen is made more reactive by a para substitution, the nitrogen reacts more strongly toward the enzyme. The second model depicts the mutagenic acridine binding to the repair enzyme at the ring nitrogen position prior to its interaction with DNA. This inhibitor-enzyme complex may alter the enzyme configuration and, thus, the DNA binding site on the enzyme. This alteration may prevent DNA-enzyme interaction or impair the function of the enzyme once it is bound to the DNA. In the case of the non-mutagen, the methyl group on the ring nitrogen prevents the enzyme-acridine interaction, and the acridine-DNA interaction does not impair enzyme function.

The feasibility of a third model in which acridines compete with the enzyme at the same DNA binding site has been eliminated by the fact that acridines vary in ability to inhibit dark repair. Riva's findings (37) showed that all acridines interact with DNA to the same extent. Thus, mutagenic and non-mutagenic acridines

should compete equally well with the enzyme on the DNA and should inhibit enzyme-DNA complex formation with the same efficiency. Consequently, similar repair inhibition would be expected for both types of acridines. However, the experiments with E. coli B demonstrated that 10-methylacridinium chloride did not inhibit dark repair and 9-aminoacridine hydrochloride did.

It was assumed that the drug effects on post-irradiation survival in E. coli B were the direct consequence of inhibition of this organism's dark repair enzyme system. This hypothesis was supported by the experiments using the two DNA repair mutants E. coli B_{s-1} and E. coli B/r. The incisionless mutant, E. coli B_{s-1} was chosen because it fails to initiate DNA repair, and therefore should show no response to repair-inhibitory acridines. This in fact was the case when (Figure 8) the post-irradiation survival of E. coli B_{s-1} was studied in the presence of 1.0×10^{-6} M 9-aminoacridine hydrochloride.

E. coli B/r, whose radiation resistance is a direct consequence of its ability to repair thymine dimers using the dark repair system, was chosen for study to offer more evidence for acridine inhibition of DNA repair. If the acridines inhibit post-irradiation survival in E. coli B/r at the same drug concentration as in E. coli B, then this is supportive that DNA repair is indeed the process being inhibited. Again the results were as predicted

and 1.0×10^{-6} M 9-aminoacridine hydrochloride inhibited post-irradiation survival in E. coli B/r (Figure 9).

From the studies with E. coli B, it was demonstrated that mutagenic acridines inhibited the overall process of dark repair. However, since DNA repair is a multi-enzyme process involving four distinct enzymatic reactions, the possibility exists that the drugs might be exerting their inhibitory effect on a single enzyme. E. coli ts-7 offered a direct means of determining if the ligase step of repair was being inhibited by the drugs.

Several differences in drug response were noted when this organism's survival was compared to that of E. coli B. The most obvious difference was that the effective drug concentration for inhibition of repair was 10 times greater than that required for inhibition in E. coli B. Since E. coli ts-7 has undergone many mutations, it is conceivable that during these events the cell wall permeability to these compounds has been altered and yet not been detected.

The second difference was that 9-aminoacridine hydrochloride caused a hundred-fold greater lethality in E. coli B than in E. coli ts-7 growing on enriched media. However, some of this difference could be due to the longer generation time of E. coli ts-7 when grown at its optimum temperature of 25°C as compared to the shorter generation time of E. coli B grown at its optimum temperature of

37°C. Another possible explanation may be that the rate-limiting step of repair has been altered in E. coli ts-7 such that acridine no longer is able to inhibit as efficiently as it did in E. coli B. This change in the rate-limiting step of repair might have been the result of two factors: 1) an inherant alteration, i.e., mutation, and 2) the presence of an excess of preformed growth compounds which may affect the rate-limiting step of excision repair.

An important observation in the E. coli ts-7 studies was that 9-aminoacridine hydrochloride inhibited dark repair to the same extent at 25°C as it did at 40°C where the ligase deficiency mutation manifests itself. From these results one must conclude that the ligase enzyme step is not the distinct site of inhibition of the mutagenic acridines.

The results from the experiments with E. coli B and the mutants are as follows. The studies using E. coli B demonstrated that mutagenic acridines inhibit the post-irradiation survival of the organisms. The two control experiments using E. coli B_s-1 and E. coli B/r added specific evidence that DNA repair was the process being inhibited by the mutagenic acridine. As was predicted, E. coli B_s-1, an incisionless mutant lacking repair, showed no decline in post-irradiation survival in the presence of the drug. E. coli B/r, a highly radiation resistant mutant, displayed a decrease in post-irradiation survival in the presence of the drug. Furthermore,

the experiments on E. coli ts-7 demonstrated that the ligase step of repair was not the single step being inhibited by the acridines. The effect of 9-aminoacridine hydrochloride on the polymerase step of dark repair has not specifically been investigated. Taking advantage of the fact that an E. coli mutant has been isolated which has lost essentially all of its repair polymerase enzyme (13,17) the drug effect on this step of repair could be investigated. A study of this type would provide more information in determining if a single step of repair is being inhibited by acridines.

In summary, it has been shown that the mutagenic acridines and ethidium bromide inhibit excision repair in E. coli B. There was a definite correlation between mutagenicity and repair inhibition, i.e., the strongest mutagens were the strongest repair inhibitors and the non-mutagen was not an inhibitor of repair. Thus, our results support the Streisinger model, which attributes frameshift mutations to errors in repair precipitated by the presence of acridines. However, in the studies reported here, the optimum concentration needed to inhibit dark repair was only a fraction of the concentration required to induce frameshift mutations. This concentration differential may be explained by two factors: 1) the biological systems used to investigate frameshift mutations are different from those used to study inhibition of dark repair, and 2) in the case of frameshift mutations, the greater

concentration of acridine may be acting to inhibit processes other than repair, i.e. replication.

V. SUMMARY

The effects of mutagenic and non-mutagenic acridines and ethidium bromide on post-irradiation survival of DNA repair mutants of E. coli were investigated. Mutagenic acridines (9-aminoacridine hydrochloride and acridine orange) as well as ethidium bromide were inhibitors of dark repair in E. coli B, and the non-mutagenic acridine, 10-methylacridinium chloride, did not inhibit dark repair. A positive correlation was found between the relative mutagenicity of the acridines and their ability to inhibit dark repair, i.e., strong mutagens were strong repair inhibitors.

The effects of 9-aminoacridine hydrochloride were also studied in E. coli B/r, a radiation resistant strain and E. coli B_{s}-1 a UV sensitive organism. In E. coli B/r 9-aminoacridine hydrochloride inhibited dark repair at the same concentration that inhibited E. coli B. The drug had no effect on the post ultraviolet survival of E. coli B_{s}-1. Since this organism lacks the incision step of repair, which means the dark repair is never initiated, its lack of response to acridines was as expected. This was taken as additional evidence that dark repair was the process being inhibited by the drugs in E. coli B.

E. coli ts-7, a mutant of E. coli TAU-bar with a temperature

sensitive ligase enzyme, was used to determine if the ligase step of dark repair was the site of inhibition by acridines. The effective drug concentration for these organisms was 10 times that required for inhibition in E. coli B. Experiments on the effect of 9-aminoacridine hydrochloride on repair in E. coli ts-7 demonstrated that the ligase enzyme was not the enzyme specifically inhibited by the drug. Control experiments using E. coli TAU-bar required the same drug concentration for inhibition as E. coli ts-7 and showed the same degree of inhibition by the drug of post-UV survival.

Thus, a model for the regulation of dark repair in E. coli dark repair mutants was proposed in which the major role of acridines in frameshift mutations was directly related to inhibition of dark repair. This inhibition may be due to the drug effect either on the overall repair process or on a single enzymic step of repair. The studies with E. coli ts-7 eliminated the ligase step as the single step of repair inhibition. Acridines may bind to DNA by means of intercalation. However, intercalation might be essential but not sufficient for acridine inhibition of dark repair. After binding to DNA, the lack of specific blocking groups, i.e., methylation, on the ring nitrogen of the mutagenic acridines allows them to inhibit selectively the function of repair enzymes. Although non-mutagens bind DNA to the same extent, they have a methyl group blocking the nitrogen in the acridine ring which allows the repair

process to proceed unimpeded.

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