L-TRYPTOPHAN PHOTOPRODUCT: EFFECT ON DNA REPLICATION

AND DNA REPAIR IN <u>ESCHERICHIA</u> <u>COLI</u>

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by

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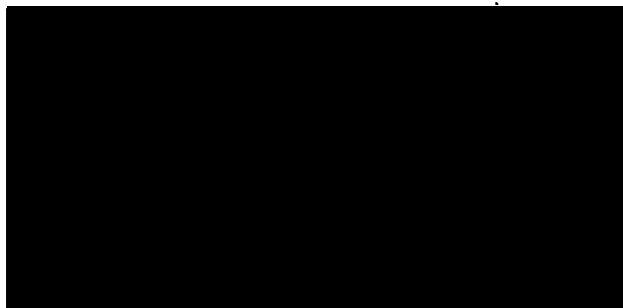A. Eisenstark   Dissertation Supervisor

The undersigned, appointed by the Dean of the Graduate Faculty, have

examined a thesis entitled

THE EFFECT OF NEAR-UV PHOTOPRODUCTS OF L-TRYPTOPHAN ON

DNA REPLICATION AND DNA REPAIR IN ESCHERICHIA COLI

presented by   GEORGE H. YOAKUM

a candidate for the degree of DOCTOR OF PHILOSOPHY

and hereby certify that in their opinion it is worthy of acceptance.

ACKNOWLEDGEMENTS

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DEFINITION OF ABBREVIATIONS

Classification	Definition
Genetic-	Escherichia coli
uvrA,B,C	Three descretely mappable genes controlling incision during excision repair of UV damage.
hcr	Mutation reducing the reactivability of irradiated page (host cell reactivation).
recA,B,C	Three genes involved in recombination (genetic).
exr	X-ray sensitive mutant (reduced in recombination).
polA1	Mutant deficient in DNA polymerase I activity.
polA,B,C	Three genes which code for DNA polymerase I, II, III.
lig$_{ts}$7	Temperature sensitive ligase mutant.
Biochemical-	
PR-enzyme	Enzyme required to enzymatically photoreactivate UV damage to DNA.
DNA polymerase I,II,III	Enzymes (3) coded for by polA,B,C genes required for DNA repair and replication.
ss-breaks (DNA)	DNA single strand breaks as detected on alkaline sucrose gradients.
ss-genome (DNA)	12.5×10^8 dalton molecule of single strand DNA.

Classification	Definition
"Okazaki fragment"	1000 nucleotide piece of newly replicated DNA.
"chase"	Following the fate of molecules or events with time.

Chemical-

MMS	Methylmethane sulfonate
EDTA	Ethylenediaminetetraacetic acid
Tris	Hydroxymethyl aminomethane
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
NADH	Diphosphopyridine nucleotide

I. INTRODUCTION

This study was stimulated by two important discoveries. The first of these came to a focus when it was observed that recombinationless (rec) mutants of bacteria were readily killed by near-UV irradiation. Recognition of the deleterious effects of near-UV has a long history (see REVIEW OF LITERATURE), but the availability of the particularly sensitive rec mutants allowed one to ask direct questions about the chromophore (the first molecule altered by the near-UV energy), and the target (the cellular entity altered to produce the observed biological effect), involved in this radiation sensitivity.

The second discovery was that the amino acid tryptophan, when irradiated with near-UV energy, yields a photoproduct that is lethal for rec mutants. Thus, a specific chromophore was identified.

These discoveries set the stage for studies on the effect of the chromophore on the target. After many exploratory trials, three areas were chosen for experiments: (1) the effect of the tryptophan photoproduct (TP) on DNA replication and cell division, (2) the effect of TP on DNA repair, and (3) interaction of TP and monochromatic 365 nm radiation on bacteria.

The last of these brings up a very important point. While the problem began with the study of a simple chromophore, and possibly a simple target, the biological effects of broad spectrum near-UV irradiation may be far more complex, with involvement of more than one chromophore.

A second complexity is the nature of the target, the DNA of the bacterial cell. The processes of replication, recombination and DNA repair involve a number of enzymes and other biomolecules that act in concert with each other. In order to evaluate the specific experimental results of this dissertation, it was decided that a thorough analysis of the literature on DNA repair and near-UV photobiology of near-UV irradiation was necessary.

Reported in the dissertation is the fitting together of new experimental results with many ideas of others. They support the conclusions that: (1) TP can interrupt the gap-closing process in DNA repair, (2) TP can uncouple DNA replication from cell division in recA mutants by inhibition of replication gap closing, and (3) TP acts as a near-UV chromophore which photosensitizes DNA to 365 nm radiation, enhances 365 nm lethality, and enhances broad spectrum near-UV mutation.

II. LITERATURE REVIEW

A. DNA REPAIR

1. Repair of Radiation Damage

The first indication that cells might have the ability to recover from otherwise lethal radiation damage was the observation that minor changes in the handling of cells (e.g., media, temperature, etc.) could profoundly effect the number of survivors in the population. Hollander and Claus, in 1937, found that fungal spores could survive higher doses of UV if they were held in water or salt solution after irradiation (1). Our understanding of this ability to recover from radiation damage became clearer when the phenomenon of liquid holding recovery was defined in 1949 by Roberts and Aldous who reported that the UV survival curve of Escherichia coli B could be dramatically altered by incubation of the cells in a medium without a carbon source before plating on nutrient agar (2). The cells' ability to demonstrate liquid holding recovery has been shown to require specific (uvr$^+$) genes (3, 4). Thus, holding wildtype E. coli B (5) and certain recombination deficient (rec) strains of E. coli K12 (6) in nonnutrient media enhances the effect of the excision repair process and

leads to increased survival of cells.

    The initial interpretation of diverse shapes of radiation survival curves called upon multiple targets (or multiple hits on targets) to explain the shoulder observed for more resistant strains. However, these theories did not account for certain observations. Under target theories, closely related mutants of E. coli would not be expected to have markedly different numbers or types of target molecules, i.e., they should have similar survival curves. But since the survival curve for radiation-sensitive strains of E. coli B/r were very different from that of wildtype, the theory was reinterpreted. Instead of a difference in targets, the capacity of these cells to repair radiation damage was implicated as the basis of the difference (7). Thus, the current concept is that the shoulder of a survival curve represents the dose range within which cells can repair radiation-induced damage to an extent that allows the cell to survive. At higher doses, where killing becomes nearly exponential, either the repair systems have been inactivated or the number of lesions in the DNA have saturated the capacity of the repair system to overcome these damages.

    The isolation of the highly sensitive E. coli B_{s-1} strain, (8) and the demonstration that this strain was not capable of reactivating irradiated bacteriophage (host cell reactivation) (9), suggested that this mutant was

deficient in the ability to repair radiation-damaged DNA.
Mutants which were unable to show host cell reactivation
(hcr) were also isolated in E. coli K12. Mapping studies
of these mutants indicated that they were the result of
mutation at any one of three widely spaced genetic loci
(uvr A, B, C) (10, 11, 3). The biochemical characteriza-
tion of the deficiencies in these mutants established the
presence and importance of enzymatic mechanisms for the
repair of radiation induced damage in DNA (12, 13).

2. Mechanisms for the Repair of Damaged DNA

Three methods of DNA repair have been documented.

2.1 Restoration of the damaged part of the mole-
cule to a functional state in situ. This may result from
the spontaneous "decay" of the damage to an innocuous
form, e.g., dehydration of pyrimidine photohydrates, the
chemical recombination of X-ray induced free radicals (14),
or the nonenzymatic photoreversal of pyrimidine cyclobutane
dimers (15, 16). A biologically important type of in situ
repair of UV induced cyclobutane type pyrimidine dimers
involves the indirect photoreversal of UV damage by
exposure to near-UV light (17, 18). Studies of indirect
photoreactivation (PR) with in vitro systems later estab-
lished this as an enzymatic process which requires the
presence of cyclobutyl pyrimidine dimers, photoreactivating
enzyme (PR enzyme), and activating light between 310 and
440 nm wavelength (19, 20). A recent and comprehensive

review of enzymatic photoreactivation has been presented by Harm et al. (21).

2.2 Removal of damaged DNA in one strand of the DNA molecule followed by the replacement of this segment with undamaged nucleotides to restore the damaged segment of DNA to its original sequence. This mechanism of repair depends on the function of a discrete set of genes in the repair pathway; it is called the excision-repair system. In contrast to the light requirement for photoreactivation, an important feature of the excision-repair system is that damaged DNA can be repaired in the dark. The first experimental evidence for the molecular repair of UV damaged DNA in the dark was provided by R. B. Setlow and co-workers (13, 22). They found that UV-resistant strains of bacteria (E. coli B/r) recovered from the inhibition of normal DNA replication following UV-irradiation, while a UV-sensitive strain (E. coli B_{s-1}) could not resume normal DNA replication after UV-irradiation (22). The mechanism of this recovery was revealed when it was shown that the resistant strain (but not the sensitive strain) released pyrimidine dimers from its DNA during dark incubation following UV-irradiation (22). Similar results were soon reported for resistant and sensitive strains of E. coli K12 and a repair mechanism was postulated (13) in which defective regions in one of the two DNA strands could be excised and subsequently replaced with undamaged nucleotides using

the undamaged portion of the DNA as a template. This
mechanism has turned out to be of general and widespread
significance for the repair of various types of structural
damage to the DNA of cells throughout the evolutionary
spectrum. The most important requirement for any particular
structural damage to enter into the excision repair pathway
is that the damage must be in only one strand of the DNA,
leaving an undamaged template to provide the information
for the resynthesis of the excised segment of DNA.

2.3 Structural damage to both strands of DNA in
close proximity, or to only one strand of a single
stranded region, requires genetic recombination with an
undamaged homologous duplex to complete the repair of the
damage. This repair pathway can function apart from the
uvr excision repair system and is called the recombination
repair system. Several lines of evidence have suggested
that excision-repair is not the only mechanism by which
cells can repair radiation damage to their DNA in the
dark. The first indication of this was the observation that
bacterial cells deficient in both excision repair (uvr) and
recombination repair (rec) were much more sensitive to UV-
irradiation than cells carrying either mutation alone (23).
This suggested that the ability to carry out genetic
recombination might be important to the repair of radiation
damage. The observation that UV-induced pyrimidine dimers
do not permanently block DNA synthesis in excision defec-

tive cells (24, 25) (DNA synthesis is blocked in E. coli B$_{s-1}$, which is a uvr exr double mutant), raised the question of the integrity of the new DNA which had been replicated while the damage was still present. After UV-irradiation uvr cells synthesize DNA which has discontinuities (as determined by alkaline sucrose gradient), the mean length of which approximates the distance between pyrimidine dimers in the parental strand (24). This discovery, together with the observation that these discontinuities disappear when the cells are incubated for a time provided the first physical evidence of recombination repair (24, 26). Recent experiments in which the parental DNA is density labeled with 5-bromouracil or ^{13}C and ^{15}N before the cells are irradiated with UV, and then incubated for a time, have shown that parent-daughter exchanges do occur in uvr$^-$ (27) and uvr$^+$ (28) strains of E. coli K12 following UV-irradiation. These experiments provide further evidence of recombinational repair of UV-damaged DNA.

3. Steps in the Excision-Repair Pathway

3.1 Recognition.

Figure 1 is a schematic representation of the postulated steps for the excision repair of damaged DNA (14). The first step in the repair process is the recognition of the damaged region in the DNA. The excision-repair system is capable of recognizing and repairing structural

defects in DNA which do not result from UV-irradiation.

Treatment of bacteria with the bifunctional alkylating

agent nitrogen mustard, which primarily attacks the 7-

nitrogen position of guanidine, causes the cell to

demonstrate repair replication (29). It has also been

shown that repair replication occurs following exposure

of bacteria to the mutagen nitrosoguanidine (30), or

following thymine starvation of a thymine requiring

strain (31). Evidence that the repair of still other

kinds of damage involves the excision repair system comes

from studies of $\underline{uvr}^+$ and $\underline{uvr}^-$ cells damaged by mitomycin C

(33), X-irradiation (34) and psoralen plus near-UV light

(35, 36). The ability to recognize many kinds of struc-

tural damage to the DNA molecule suggests that the recogni-

tion step may be associated with a change in the secondary

structure in the phosphodiester backbone of the DNA.

Hanawalt and Haynes (29, 36) have suggested that the

damage recognition step may be equivalent to threading the

DNA through a close fitting "sleeve" which gauges the

secondary structure of the molecule. This is the "sleeve"

which appears in (I) of Figure 1.

The repair of damage which includes a single

strand break in the DNA, such as in X-irradiation, ^{32}P-

decay, ^{3}H-decay, or methylmethanesulfonate, may not require

the recognition or incision steps, and repair might proceed

with the next step (37, 38). This would explain why some

Figure 1.--Schematic representation of the postu-

lated steps in the excision repair of damaged DNA. Steps

I through VI illustrate the "cut and patch" sequence. An

initial incision in the damaged strand is followed by local

degradation before the resynthesis of the region has begun.

In the alternative "patch and cut" model the resynthesis

step III begins immediately after the incision step II and

the excision of the damaged region occurs when repair

replication is complete. In either model the final step

(VI) involves a rejoining of the repaired section to the

contiguous DNA of the original parental strand (157).

EXCISION-REPAIR

I. Recognition

II. Incision
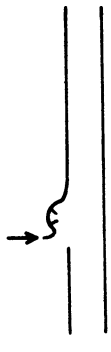
III. Excision
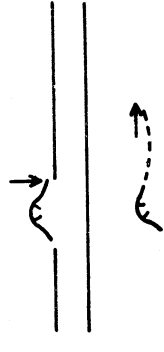
IV. Degradation

V. Repair replication
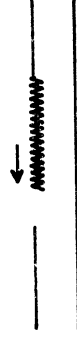
VI. Rejoining
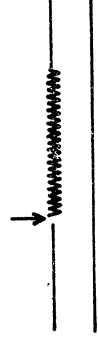
Alternate Steps
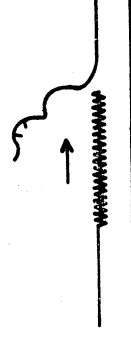
III'. Repair replication

IV'. Excision

V'. Degradation

IIa

UV-Endonuclease ± Mg^{++}

IIIa

Mg^{++} UV-Exonuclease

Mg UV-Exonuclease
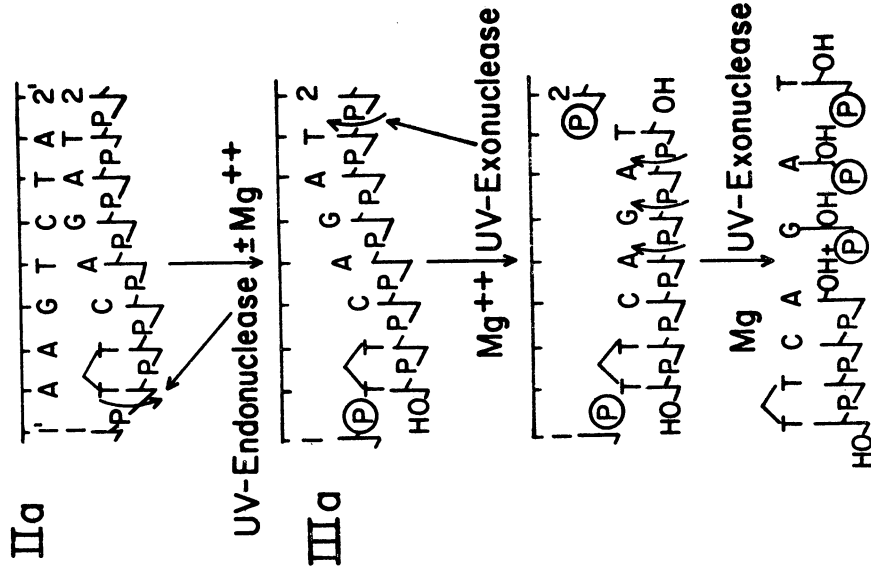
Figure 1

UV-sensitive mutants, presumably deficient in the recognition and/or incision steps, do not appear to have a parallel sensitivity to X-irradiation. In fact these uvr mutants are no more sensitive to X-irradiation than wild-type cells (37).

While the consequences of a pyrimidine dimer entering the replication complex was once considered a question of great importance (39), the elucidation of the post-replication (rec) repair system has resolved this problem. If a dimer passes through the replication complex, the dimer will be opposite a strand gap; this results in damage to both strands and requires recombinational repair. This suggests that the recognition mechanism of the excision repair system probably does not also function by relaying "messages" to the replication complex to stop DNA replication and avoid the impending disaster of a dimer in the replication fork, as was once thought to be the case (39). Thus, the probable function of the recognition mechanism is to simply locate single strand discontinuities in the DNA molecule that result from various kinds of structural damage, and then to initiate excision-repair.

3.2 Incision.

The second step portrayed in Figure 1 (step II) is the incision step of excision-repair. Following the recognition of damage in the DNA, a necessary prerequisite

to the excision of the damaged region is the production of an endonucleolytic incision near the damage. This incision step precedes the exonucleolytic removal of the damaged region, and is required for efficient levels of excision-repair of damaged DNA (40). In E. coli, excision-repair is dependent on the uvr gene products (3). The uvrA and uvrB gene products appear to be necessary for the DNA incision step (3, 40, 41, 42) while the uvrC gene product may be involved in the regulation of the incision and excision steps (40).

While attempts to purify dimer-specific nucleases from uninfected E. coli have not been successful, two nucleases which catalyze the excision of photoproducts from UV-irradiated DNA have been extensively purified from Micrococcus luteus (43). Step IIa in Figure 1 (44) represents the incision step as it occurs in vitro for reactions with purified M. luteus UV-endonuclease (44, 45). This incision is 5' to the dimer (-TT-) leaving a 5'-hydroxyl and a 3'-phosphomonoester group (44, 45). This UV-endonuclease introduces single strand breaks into irradiated DNA as described above, however, this nuclease will not attack undamaged native or single stranded DNA (43). Thus, the in vitro UV-endonuclease from M. luteus probably affects repair of radiation damage in a similar manner for this species in vivo. While the details of incision are not as clearly understood for E. coli, esti-

mates of the size of piece replaced following excision-
repair (23, 46, 47) indicate that the nick must be made
very near the damage, as has been shown for M. luteus
UV-endonuclease. Therefore, incision is a process which
involves the recognition of damage in the DNA, and is a
step which must precede the exonucleolytic removal of the
damaged portion of the DNA.

3.3 Excision.

Step III in Figure 1 represents the excision step
in the excision-repair of damaged DNA. The type of
excision depicted in step III, and IIIa, is consistent
with the "cut-and-patch" mechanism proposed for dark
repair (23). The in vitro properties of M. luteus UV-
exonuclease are shown in IIIa of Figure 1. This step is
catalyzed by the UV-nuclease; initial cleavage results
in the release of a large photoproduct-containing fragment.
As depicted in IIIa, this process leaves a 5'-phospho-
monoester on the DNA molecule and a 3'-hydroxyl on the
fragment (44). Unlike most exonucleases the UV-exonuclease
is not inhibited by the presence of UV-photoproducts in
the DNA (45). The UV-exonuclease hydrolyses unirradiated
or UV-irradiated DNA to the same extent but does not
attack native DNA or RNA (45). Denatured DNA may be
degraded from both the 3' and the 5' termini, releasing
mostly 5' monophosphates from UV-irradiated DNA (44).
This nuclease will not act on un-nicked DNA, making recog-

nition and incision the controlling steps in the "cut-and-patch" type of excision repair.

Since DNA polymerase I requires a 3'-hydroxyl on the 5'-end of the gap to initiate repolymerization (48), the presence of the 3'-phosphomonoester following the excision described for UV-exonuclease would present a block to gap repolymerization. Therefore, the initiation of repair replication may require the participation of either a polynucleotide phosphomonoesterase or an exonuclease-III type digestion to prepare the gap for the repair synthesis step. Repair replication would then proceed as depicted in step V of Figure 1, and finally repair would be completed by DNA ligase rejoining (49).

The "patch-and-cut" model of excision-repair (50), presented as Alternate Steps in Figure 1, may be the most accurate model for the excision-repair mode as it occurs in E. coli. Following the characterization of the 5'→3' nuclease activity of E. coli DNA polymerase (51, 52, 53), Kelly and co-workers presented a hypothetical scheme for the participation of DNA polymerase in the repair of irradiated DNA (51, 53). In contrast to the UV-endonuclease, the 5'→3' nuclease associated with DNA polymerase has an absolute requirement for native DNA as a substrate (52). Also, this exonuclease site is oriented relative to the polymerizing site so as to permit one enzyme to synthesize and hydrolyze simultaneously (52). The observation that

the 5'→3' exonuclease activity of DNA polymerase I was
not inhibited by photoproducts in UV-irradiated DNA (51),
and that native DNA was required as a substrate (52),
suggested that repair synthesis and excision might occur
simultaneously (53).

3.4 Repair Replication.

Direct physical evidence for the repair synthesis
step in the postulated scheme was provided by the studies
of Pettijohn and Hanawalt (54, 55). When 5BU was used to
label the DNA synthesized after UV-irradiation of E. coli
strain TAU-bar, the density pattern was not consistent with
the pattern for normal semi-conservative DNA replication
(23). Instead of a hybrid density band in the gradient,
there was a band of DNA with a density intermediate to
that of normal (unreplicated parental DNA with no BU)
and hybrid DNA (half light parental and half BU labeled
daughter DNA) (47). While this intermediate density DNA
is not consistent with the semi-conservative mode of DNA
replication, it is consistent with the resynthesis of
segments of DNA which have been removed by the excision-
repair system.

The physical size of the segments of DNA replaced
by repair-replication, after the excision of damage from
UV-irradiated DNA, was first estimated by Pettijohn and
Hanawalt (54, 55). Using isopycnic CsCl centrifugations,
they observed 1% bromouracil (BU) substitution in 15TAU

DNA and estimated (assuming all dimers were excised) that
approximately 20 nucleotides were incorporated for each
dimer excised. However, later work by Cooper and Hanawalt
(28) indicated that the size of repaired regions varied
over a wide range. Since isopycnic centrifugation techni-
ques were used for the original size estimates these
calculations could be highly susceptible to error (56).
Ley and Setlow (56) have employed a new technique based
on the 313 nm photolysis of BU containing DNA to estimate
the size of repair replicated regions following the
excision-repair of UV-damage. Since this technique does
not employ the use of isopycnic gradients it is much less
susceptible to error introduced by the differences in the
size of various repaired segments (56). Based on the
calculations of Ley and Setlow (1972), the average size of
the segments replaced by repair replication following
excision of UV-photoproducts is 12 to 20 nucleotides (56).

Strains of E. coli K12 carrying a polA1 mutation
are deficient in a DNA polymerase I activity (57) and also
show a slower rate of excision of UV-induced thymine
dimers than the related wildtype strain (58). The polA1
strain is partially deficient in repairing incision breaks
in DNA produced during the excision repair process (59,
60). Most of the repair of incision breaks which occurs
in the polA1 mutant appears to require DNA polymerase III,
since this repair is much reduced in a polA1, polC double

mutant (61). Thus, both DNA polymerase I and III appear to be involved in the DNA resynthesis step of the excision repair process.

Masker et al. (62) have shown that DNA polymerase II [deficient in polB mutants (63)] is involved in UV-induced repair replication in toluene-treated cells which lack both DNA polymerase I and normal DNA replication. However, no excision-repair role has yet been demonstrated for DNA polymerase II in vivo. It seems that DNA polymerase II is not involved in the excision-repair of UV or X-ray induced damage in vivo since wildtype or polA1 cells which contain a polB mutation are no more sensitive to UV or X-irradiation than related polB$^+$ strains (63, 64). Thus, the repair replication observed for DNA polymerase II in toluenized cells represents either a trivial substitution for DNA polymerase I and III in their absence, or indicates that residual DNA polymerase II still present in these mutants (63, 65) is sufficient to carry out repair replication in vivo. While this mutant (polB) seems normal in all aspects of DNA replication and repair (63, 65, 66) a cautious note should be attached to interpretation of these results since this mutant was isolated as an in vitro deficiency (66). For example, E. coli mutants which have extracts with less than 1% detectable ligase activity show normal growth and repair abilities (67), but temperature sensitive mutants have been isolated

which demonstrate an absolute phenotypic requirement for DNA-ligase activity (68, 69). Therefore, phenotypic characterization of polB may not be feasible until a deletion mutant (possibly a temperature-sensitive mutant) is isolated for the polB gene. It would be premature to conclude that the characterization of the existing polB mutants excludes DNA polymerase II from a role in DNA repair.

3.5 Rejoining.

The first evidence for the occurrence of the rejoining step in vivo is found in the work of McGrath and Williams (1966), who examined the molecular weight of single-strand DNA fragments following gentle lysis of bacteria on top of alkaline sucrose gradients (70). Using this technique, they followed both the appearance of UV-induced excision-repair breaks and the rejoining of the repaired segments. Gellert, in 1967, (49) isolated a mutant of E. coli which was unable to demonstrate ligase joining of DNA in cell extracts in vitro. However, this mutant and other ligase deficient mutants, demonstrate normal growth and irradiation resistance in vivo. This was mistakenly taken as evidence that ligase had no significant role in DNA replication or repair in vivo. Modrich and Lehman (71), isolated a temperature sensitive mutant in E. coli $\overline{TAU}$ ($lig_{ts}7$) with a temperature sensitive ligase activity in vitro. The $lig_{ts}7$ mutant of $\overline{TAU}$

is sensitive to UV-irradiation and to treatment with methyl methane sulfonate, even at permissive temperatures, indicating that DNA ligase is involved in the repair of UV and MMS damage to DNA $\underline{in}$ $\underline{vivo}$ (71). This has been unambiguously established by the genetic transfer of the $\underline{lig}_{ts}7$ mutation from the $\underline{E}$. $\underline{coli}$ $\overline{TAU}$ background to $\underline{E}$. $\underline{coli}$ K12 without the loss of the $\underline{lig}_{ts}7$ phenotype (69).

The scheme in Figure 2 shows the mechanism of the reaction catalyzed by $\underline{E}$. $\underline{coli}$ DNA ligase (72). The synthesis of phosphodiester bonds in duplex DNA by DNA ligase of $\underline{E}$. $\underline{coli}$ appears to proceed in a sequence of three steps involving two covalent intermediates (72) (Figure 2). Ligase-adenylate, in which AMP is linked to the ε-amino group of a lysine residue of the enzyme through a phosphoamide bond (73), is the produce of incubation of the enzyme with DPN in the absence of DNA (74, 75). The second intermediate is DNA-adenylate, in which AMP is bound in a pyrophosphate linkage to the 5'-phosphoryl terminus of the DNA to be joined (76, 77). Finally the closing reaction is completed, as shown in Figure 2, with the release of AMP (73).

$\underline{E}$. $\underline{coli}$ appears to contain about 300 DNA ligase molecules per cell (78). A turnover number of 25 min^{-1} at 30° therefore indicates a capability of sealing 7,500 single strand breaks per minute per cell (72). Assuming that both strands of $\underline{E}$. $\underline{coli}$ replicate discontinuously

Figure 2.--Mechanism for the reaction catalyzed
by the E. coli DNA ligase (72).
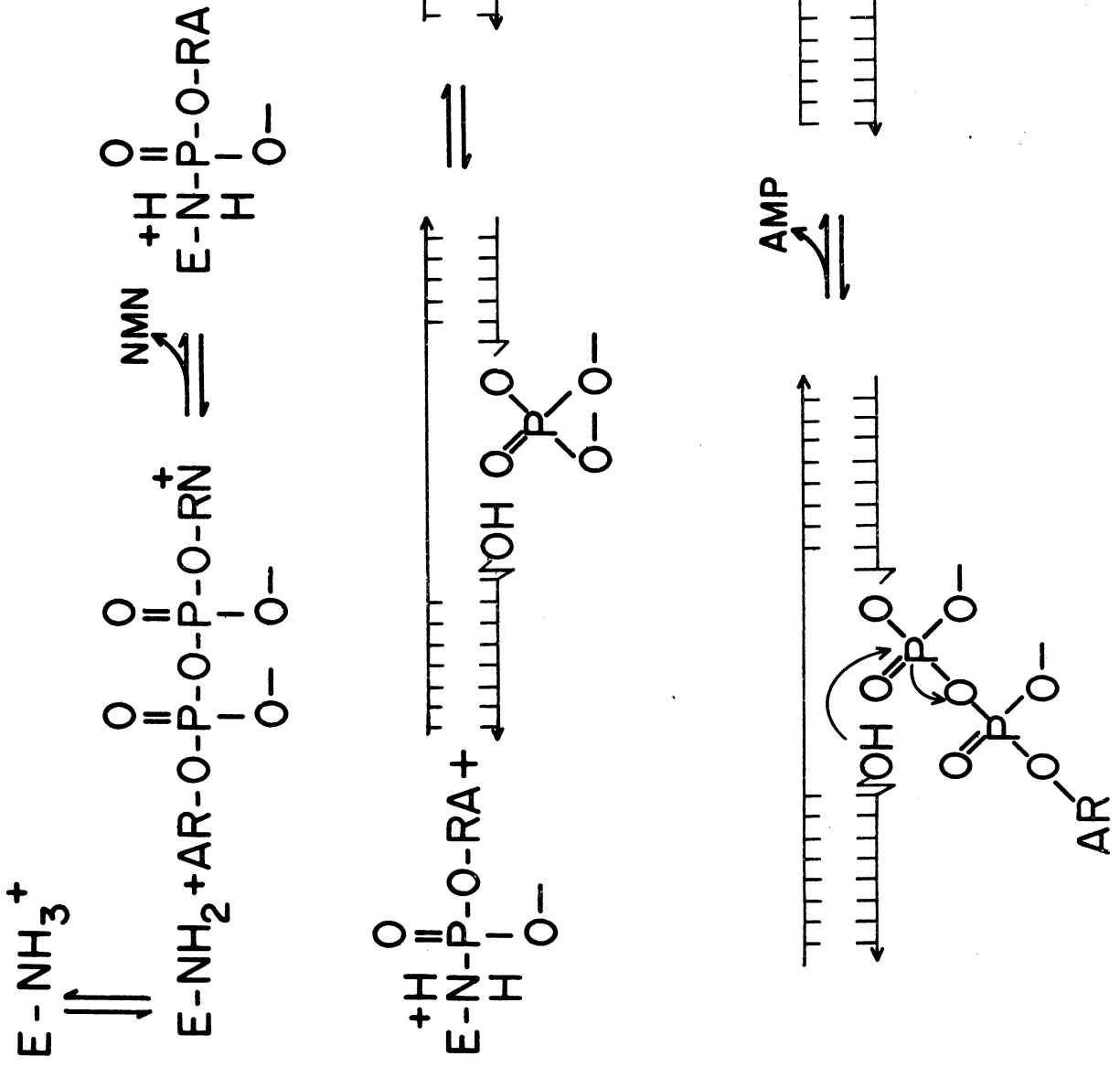
Figure 2

(69, 79), that the time required to replicate the chromo-
some at 30° is about 65 min (80-82), and that the average
length of an "Okazaki fragment" is about 1000 nucleotides
(79), about 200 sealing events per cell would be sufficient
to join replicative intermediates within a replication
period (72). Thus, less than 3% of the normal amount
of ligase activity should be sufficient for DNA replica-
tion (72). This is consistent with the observation that,
while E. coli lig$_{ts}$7 extracts have only 1-3% of the
quantity of wildtype ligase activity even at permissive
temperatures, this mutant grows normally at permissive
temperatures (although the joining rate for nascent DNA
is depressed). The apparent presence of a 95-97% excess
of ligase joining capacity suggests either that E. coli
normally maintains the capability to close a tremendous
number of repaired breaks in DNA, and/or the cell must
close many breaks which occur during normal semi-conserva-
tive DNA replication (i.e., some such breaks might result
from nuclease mediated "unwinding" of DNA to be replicated).

The discovery by Town et al. (1972) (83), of an
"ultrafast" X-ray repair process which is independent of
DNA polymerase I activity provides the first direct ex-
perimental evidence concerning the role that this large
DNA-ligase mediated break closing capacity plays in the
survival of cells following the introduction of X-ray
damage. The observation that biological systems are much

more sensitive to X-irradiation in the presence of oxygen (84) is a striking and well documented effect. The involvement of any DNA repair process in this effect was uncertain. However, Town et al. have shown that the X-ray oxygen-nitrogen break ratio is 3.2 for the yield of single strand breaks in E. coli cells (83). This difference in break yield could account for the difference in sensitivity in the absence of oxygen. The repair of X-ray induced single strand breaks in DNA following irradiation in nitrogen occurs very rapidly even at $0^{\circ}C$, and is inhibited by EDTA or temperature shock pretreatment of the cells (i.e. cold shock) (83). The special repairability of X-ray damage which occurs in the absence of oxygen is probably the result of larger yields of "clean-breaks" that can be repaired by ligase activity alone. Although this "ultrafast-repair" has not been conclusively shown to be ligase dependent (either genetically or biochemically), the isolation of "tighter" ligase deficient mutants may make conclusive experiments possible. Thus, ligase-mediated closing of DNA single-strand breaks is a process important to the viability of cells. It is required both for the semi-conservative replication of DNA and the completion of repair of damage induced in DNA by UV, X-irradiation, etc.

3.6 Separate "Branches" of Excision-Repair

Youngs, Schueren, and Smith have recently found evidence that the excision repair of UV-induced damage to DNA can be operationally divided into two branches (see Figure 3). One of these requires growth medium and a second does not (85). In wildtype and polA1 strains, they found that more extensive repair of incision breaks occurred when the cells were incubated in growth medium than in buffer. Also, chloramphenicol was observed to inhibit this medium-dependent repair. DNA polymerase I activity does not seem to be involved in the growth medium-dependent, chloramphenicol sensitive, branch of excision repair (85). However, the absence of DNA polymerase I activity reduced the efficiency of the medium-independent branch of excision repair to about 25% of the repair of incision strand breaks measured in the related wildtype strain (85). This indicates that DNA polymerase I is responsible for approximately 75% of the repair synthesis (and possibly for 75% of excision) observed in buffer conditions.

The recA56, recB21, and exrA strains appear to be deficient in the growth medium-dependent branch of excision repair (85). Incubation of these strains in a growth medium does not allow the cells to complete any repair events that were not repairable under medium independent conditions (85). Thus, the growth medium-dependent repair

Figure 3.--The separate branches of excision repair, and a scheme showing the possible mechanism by which medium dependent repair is initiated (85).

UV DAMAGED DNA

uvr

SINGLE-STRAND BREAKS IN DNA

growth medium
independent

growth medium
dependent

exrA
recA
recB
recC
polC polB?
CAP

polA

polC
polB?

REPAIRED DNA

BRANCHED
EXCISION
REPAIR

1.

incision
excision (medium-independent)

2.

rec, exr,
pol II? pol III (medium-dependent)

3.
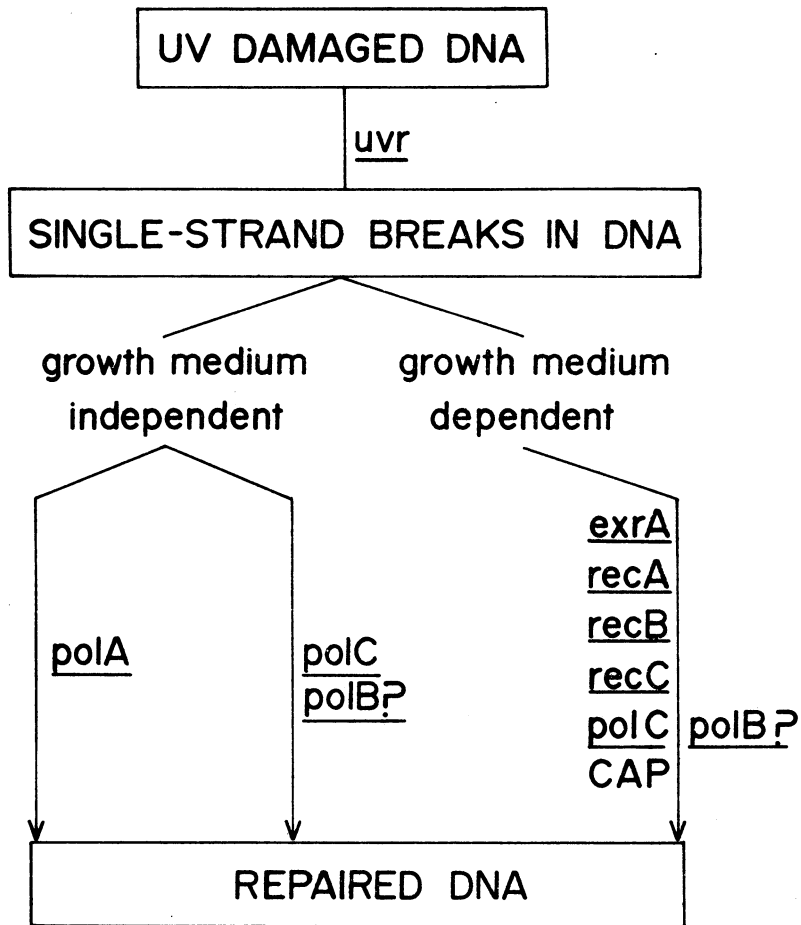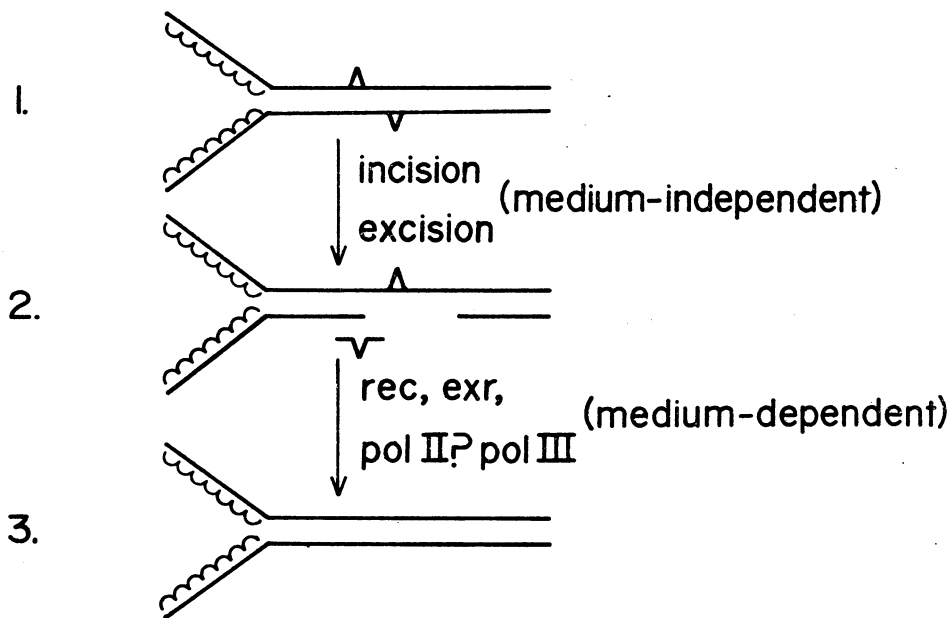
Figure 3

process appears to require the $\underline{rec}^+$ $\underline{exr}^+$ genotype (85).
The $\underline{polA1}$ strain appears to be deficient only in the $\underline{uvr}^+$
gene dependent excision repair process (86), and most of
the repair of incision breaks in the DNA of this strain
appears to be $\underline{pol\ III}$ dependent (61, 87). However, it
should be noted that this interpretation is based on the
$\underline{in}$ $\underline{vivo}$ phenotypic characteristics of $\underline{polB}$ mutants isolated
for a deficiency in an $\underline{in}$ $\underline{vitro}$ activity (as discussed
previously). Therefore, a portion of the repair associated
with $\underline{pol\ III}$ could be a $\underline{pol\ II}$ function.

Figure 3 is a scheme of the gene pathways for the
medium-dependent and medium-independent branches of $\underline{uvr}^+$
controlled excision-repair. The lesion enters the exci-
sion repair pathway through incision near a pyrimidine
dimer. The growth medium-independent branch of excision
demonstrates a 75% dependence on DNA polymerase I activity.
The remaining 25% of incision breaks repaired in medium-
independent conditions are repaired by DNA polymerase III
(and possibly polymerase II). Recent $\underline{in}$ $\underline{vitro}$ experiments
with $\underline{B}$. $\underline{subtilis}$ $\underline{pol\ III}$ indicate that this DNA polymerase
also can act as a DNA exonuclease (Cozzeralli, personal
communication). If $\underline{B}$. $\underline{subtilis}$ $\underline{pol\ III}$ nucleolytic activ-
ity is similar to $\underline{E}$. $\underline{coli}$ $\underline{pol\ I}$ nuclease, this enzyme may
be able to excise damaged regions in a similar manner.
The involvement of $\underline{polB}$ gene product in medium-independent
or medium-dependent repair is not clear at this time. The

growth medium-dependent branch of repair involves gene
products which are important to recombination repair (85).
Therefore, the medium-dependent repair may involve a
recombination repair step for completion. The scheme at
the bottom of Figure 3 proposes a hypothetical mechanism
by which branched excision repair might occur. The over-
riding features of this proposed mechanism are: a) the
medium-independent steps are incision and excision, b) if
excision of one strand causes a gap opposite a dimer (by
chance of nearest occurrence) then step 3 is required,
c) the repair of a dimer adjacent to a single strand gap
in the opposite strand involves a recombinational event
(step 3). Thus, the medium-dependent, chloramphenicol
sensitive, repair of UV incision breaks may involve
recombination steps as intermediates in this "branch" of
excision-repair.

4. Recombinational Repair of DNA

4.1 Genetic Recombination.

One of the most important milestones in the history
of biological sciences was the discovery by Lederberg and
Tatum (1946) of genetic recombination in bacteria (88).
Several comprehensive reviews of genetic recombination
are available [see Hayes, 1968 (89); Curtiss, 1969 (90);
and Clark, 1971, 1974 (91, 92)]. Certain strains of
bacteria (e.g., E. coli K12) are capable of a sexual
process called conjugation. During conjugation there is

a oneway transfer of genetic material from the donor
(male) to the recipient (female) bacteria. On the average,
approximately 10 to 20% of the donor chromosome is
transferred to the recipient. With a certain probability,
the homologous chromosomes will pair and recombination
will take place by breakage and rejoining to yield a
progeny cell carrying genetic information from both parents.

Although it is the central tool of genetic analy-
sis, the molecular details of even bacterial recombination
are not known. However, some simple generalizations at
the molecular level have been made to explain (partially)
the observed biological consequences of recombination
(93-95):

a) Single-strand breaks are produced in the two
DNA heteroduplex molecules.

b) Single-stranded regions are produced by
"unwinding", or nuclease activity at the single strand
break.

c) Covalent joining occurs between strands with
similar sequence (one, or both) from different DNA mole-
cules.

d) Annealing of complementary single strands
from different DNA molecules occurs during the insertion
of a single strand of DNA (e.g., transformation).

e) Repair processes (possibly involving portions
of the excision-repair pathway) convert the hybrid molecule

to a complete two stranded molecule without single-strand interruptions.

It was generally assumed that recombination during the various sexual processes of bacteria; e.g., in transformation, transduction, and conjugation, events occur via a single molecular mechanism. However, Clark (91, 92) has eloquently pointed out that there are probably several mechanisms for recombination. Recently, detailed evidence has suggested that this is indeed the case. While most of the proposed mechanisms of recombination require extensive repair synthesis (95-100), Gross and Gross (101) have observed that mutation of the polAl gene, leading to deficiency of a DNA polymerase function important to DNA repair processes, does not reduce the recombinational abilities of E. coli. They found that polAl deficient cells demonstrated about twofold less conjugational transfer of genetic markers. However, this reduction was caused by a parallel reduction of chromosome transfer and recombination was taking place at a normal rate in the polAl mutant (98). Recent work with T4 phage recombination has shown that single crossover events have the same probability of occurrence in polAl hosts as in wildtype hosts. However, double recombination events are much reduced for T4 markers incubated in polAl host cells (102). This suggests the possibility that double crossover events may introduce large enough single stranded regions

to require extensive polAl mediated repair synthesis, and provides evidence that at least two types of recombinational processes, (i.e., E. coli conjugation, and T4 recombination) take place along different (or divergent) pathways.

The isolation and characterization of mutants deficient in genetic recombination (103), and the discovery of enzymes which are the product of recombination genes (104-107) allows for the study of the molecular details of recombination mechanisms. Clark's concept of many recombinational pathways indicates that each recombinational process (e.g., transformation, conjugation, etc.) must be studied independently. Thus, an overview of recombinational processes must await the elucidation of the molecular intermediates and enzymology of each separate process. However, the recombination-repair system offers several advantages as a model system which can be used for the initial isolation of intermediate structures of recombination and characterization (both in vivo and in vitro) of recombination enzymes. The most important aspect of the recombination-repair system as a generalized model, is the fact that genetic recombination and recombination-repair require the same gene products. This suggests that recombination-repair involves similar intermediate structures and shares regulatory steps and enzymatic pathways with genetic recombination. An operationally important advantage of the recombination-repair system is that

damage which requires recombination-repair (for a detailed discussion, see Literature Review Section A. 4.) can be introduced and studied throughout the chromosome, whereas genetic recombination involves the study of highly localized regions of the chromosome. This operational difference substantially increases the total number of events which can be examined in an experiment, and consequently increases the probability of isolating intermediate DNA configurations. Since in vitro partial cell systems (i.e., toluenized bacterial cells) are only partially functional by comparison with whole cells, the ability to study a larger number of events during recombination-repair is an important advantage. Thus, recombination-repair of experimentally introduced damage provides an excellent model system with which intermediate configurations of recombining DNA can be isolated, in vitro studies of appropriate mutants might reveal the function of purified recombination enzymes with this partial cell system.

4.2 Properties of Recombination-Defective Mutants of E. coli.

Mutation at three distinct but closely linked loci results in a large reduction in the number of recombinants observed during conjugation (or transduction (108-111). These genes (recA, recB, and recC) lie within a 5% segment of the E. coli chromosome (112). Barbour and Clark have

conducted an intensive search for other genetic deficiencies which will further reduce the number of recombinants in cells carrying these mutations (91, 113, 114). Using recB recC double mutants, they have discovered several new mutable loci which can further reduce the recombination ability of E. coli. However, no mutation has been isolated which can further reduce the recombination ability of a recA deficient cell. This suggests that either mutations or combinations of mutations which lower recombination abilities below the level performed by recA mutants are lethal, or the recA gene product is a control mechanism for the major pathways of recombination.

The recB and recC gene products yield an ATP-dependent nuclease with the ability to exonucleolyticly attack double strand DNA (104-106) and degrade single strand DNA with an endonucleolytic specificity (107). While no enzymatic function has been clearly demonstrated for the recA gene product at this time, there is evidence that recA gene product acts as a negative control for recB recC nuclease. E. coli recA cells degrade a great deal more of their DNA following the introduction of damage into their DNA. In contrast, recB and recC mutants degrade less than related wildtype cells. Also, the addition of a recB or recC mutation to a recA mutant does not increase the radiation sensitivity of the cells, and inhibits the DNA breakdown observed in the recA strains

(115). Thus, in the absence of a functional recA product,
the recB recC nuclease apparently runs free of control
and degrades the DNA extensively.

There are a number of lines of evidence which
suggest that the recA$^+$ gene yields either a very pleiotro-
phic gene product, or that some or all of the recombina-
tional pathways controlled by the recA gene play heretofore
unpredicted roles in many cell processes.

The following is an abridged listing of the cel-
lular phenomenon which are affected in some way by the
recA gene product:

a) While E. coli recB or recC strains carrying
F lac$^+$ can mobilize their chromosomes with 10-50% of
wildtype efficiencies, recA reduces this to less than
10^{-4} of normal (116).

b) E. coli recA strains cannot be mutated by UV-
light while recC derivatives show an intermediate mutabili-
ty, suggesting that UV-mutation either takes place by
recombination or that mutation-fixation must be preceded
by a recombinational step (117).

c) Inhibition of DNA synthesis in recA strains
uncouples DNA synthesis from cell division and leads to
cell death (118, 119).

d) While recB and recC strains demonstrate a
nearly normal rate of spontaneous production of λ phage,
recA derivatives are depressed in the ability to produce λ

phage spontaneously (108).

e) Both recA and recB strains are sensitive to killing by the DNA synthesis inhibitor naladixic acid and 5-fluorodeoxyuracil (120). However, the recB mutant is sensitive to thymineless death (121), whereas recA mutants are not especially sensitive to thymine starvation (122). While many of the processes or phenomenon above were already thought to involve recombinational events (i.e., λ phage induction), other processes such as the coupling of DNA replication with cell division were not known to require recombination related gene products.

RecA mutants are more sensitive to UV and X-ray than are recB or recC (14, 123). The radiation sensitivity of recA results from the inability to perform postreplication-repair following UV-irradiation, whereas recB and recC mutants show no apparent deficiency in this response (124). Also, recA mutants are deficient in their ability to repair X-ray induced single strand breaks in DNA, while the recB and recC mutants show an intermediate ability to repair X-ray breaks (123). The requirement for functional recA gene product to repair sectors of both UV and X-ray damage suggests that these two classes of damage may involve similar structural features at some step in their repair which requires a recombinational step for completion.

4.3 Recombinational Repair of UV-induced Damage.

DNA synthesis can occur on UV-damaged template
DNA. Mutants of E. coli which are defective in excision-
repair alone can resume DNA synthesis using DNA containing
pyrimidine dimers for a template (24, 25). It is of
historical interest that the early isolation of radiation
sensitive E. coli B_{s-1} (8) led to the misconception that
it was impossible for UV-induced dimers to pass through
the replication fork. This interpretation resulted
when it was observed that this strain could not excise
dimers (13), and that normal DNA synthesis did not
resume following UV-irradiation (22). However, it was
later discovered that the reason for the extreme sensitivity
of E. coli B_{s-1} is that this strain is defective in both
uvr (excision repair) and exr mediated recombination-
repair. Once it was conclusively shown that dimers
could indeed be bypassed during DNA replication, experi-
ments were immediately initiated to elucidate the physical
structure of the resultant daughter DNA following synthesis
on a damaged parent template. During such experiments,
the DNA from uvr mutants labeled in their DNA prior to
or after UV-irradiation was separated in alkaline sucrose
gradients with the McGrath-Williams gradient technique
(24, 56). The single strand molecular weight of the DNA
was then analyzed by velocity of sedimentation through
the alkaline sucrose density gradient.

Rupp and Howard-Flanders observed that newly synthesized DNA labeled immediately following UV-irradiation of uvr mutants is synthesized in pieces approximately equal in size to the average distance between the dimers in the damaged parent DNA template (24). The resulting gaps in daughter DNA appear to be approximately 500-1000 nucleotides (125), and to be opposite pyrimidine dimers as tested in conjugating bacteria (26). The recent observation that photoreactivation greatly increases the rate of postreplication gap closure provides strong support for the concept that these gaps are opposite dimers and that the presence of the dimer inhibits rapid repair of the postreplication gap (126). The observation by Iyer and Rupp (125) that the size of gaps in newly synthesized DNA is approximately equal in size to an "Okazaki-fragment" has suggested the following. Since one might postulate that each Okazaki fragment has an initiating and terminating point, the presence of a dimer in the parental DNA might cause an Okazaki-fragment size "skip" during DNA replication of the damaged template (15). The observation that recA[+] restricts recB recC nuclease activity following UV-irradiation correlates with the observation that "Okazaki-fragment" size DNA-pieces are normally removed following irradiation (125). These observations are consistent with reports that newly synthesized DNA is preferentially

degraded following irradiation (127, 128, 129). Estimates
have been made of the size of the segment replaced during
postreplication recombinational-repair of 254 nm UV-
damage using 313 nm photolysis of BU containing DNA
as described previously for excision repair. This suggests
that recombination-repair regions of postreplication gaps
are approximately 1.5×10^4 nucleotides in size (130),
and that the postreplication gap (500-1000 nucleotides)
is enlarged nearly 15-fold during the recombination repair
process. This observation is also consistent with the
interpretation that the "long-patch repair" reported by
Cooper and Hanawalt is the result of recombination-
mediated repair.

   4.4 Recombinational Repair of X-ray Induced Damage.

   The terminology which describes three modes of
X-ray repair is the result of recent work of X-ray induced
single-strand breaks in bacterial DNA (83). These three
modes of repair can be described as follows: a) Type I-
Ultrafast rejoining of "clean" breaks which occur more
frequently in N_2 purged cells than in air bubbled samples.
These breaks are probably repaired by ligase activity.
b) Type II-DNA polymerase I mediated repair of single
strand breaks at room temperature in buffer with no
energy source available. This mode of X-ray may be
characterized as "Medium-Independent" and shows no require-
ment for functional $\underline{rec}^+$ genes. c) Type III--This mode of

X-ray repair is dependent on a functional recombination-
repair system and only occurs when the cells are incubated
in complete medium. It is also suggested that there may
be a requirement for DNA polymerase III activity during
this "Medium-Dependent" class of X-ray repair. While
questions have been presented concerning the involvement
of DNA ligase in Type I X-ray repair, there is enough
evidence to tentatively consider ligase activity a
requirement for this mode of break repair. The argument
against the involvement of ligase is that cold shocked
cells (which are unable to demonstrate ultrafast repair)
have been shown to leak sulfhydryl compounds. SH-
containing compounds are classical radioprotective agents
(especially in the presence of O_2) and the ultrafast
repair sector could be accounted for on the basis of
physical protection of the DNA. However, it should be
noted that the cold shocked cells probably also leak
$NADH_2$ which is required for ligase activity, providing a
scheme consistent with a ligase mediated repair function.
This controversy will probably remain active until some
conclusive experiments establish whether "ultrafast X-ray
repair" is an enzymatic repair phenomenon.

Type II X-ray repair (131) appears to be somewhat
analogous to the kind of excision-repair which occurs
during liquid holding recovery (LHR) incubation of cells
which have been damaged by UV-irradiation. The similarities

are as follows: a) Both processes are "Medium-Independent"
and occur effectively at room temperature. b) Both LHR
and Type II X-ray repair take place in recombination
deficient strains. Therefore, neither LHR nor Type II
X-ray repair requires strand exchanges and probably involve
only the repair of lesions in one strand of DNA adjacent
to an undamaged template from which DNA polymerase I can
synthesize an accurate repair segment.

Type III X-ray repair (83) apparently shares some
similarities with "post-replication repair" of UV-induced
damage to DNA. The similarities are as follows: a) Repair
of both types of damage requires the presence of an energy
source (i.e., glucose) in the post-irradiation incubation
medium for repair to occur. b) Post-replication repair
of UV-induced damage (24) and Type III X-ray repair (83)
require a functional recombination repair system; thus,
DNA strand exchanges are important to these repair pro-
cesses. Both UV and X-irradiation can damage the DNA in
a manner which results in a single strand gap opposite a
segment of damaged DNA. This would require the insertion
of a new piece of undamaged DNA from an homologous DNA
duplex via recombination (132). The current evidence for
"branched excision-repair" (56), demonstrating that there
is a medium-dependent sector of excision repair which
requires functional rec genes and a functional polC (polB?)
gene product, opens the possibility that rec repair and

excision-repair occur in some coordinated manner.

4.5 Recombinational Repair of DNA Containing Crosslinks.

Covalent crosslinks between complimentary strands of DNA in vitro, or in cells, have been detected after treatment with several agents including the following: a) nitrogen or sulfur mustards (133), b) nitrous acid (134), c) mitomycin C (135), and psoralen plus-light (136-138). Low yields of crosslinks have also been detected after exposure to UV-light (139), and ionizing irradiation (140). Animal cells growing in tissue culture maintain their ability to grow and divide after treatment with bifunctional alkylating agents that produce thousands of interstrand crosslinks per cell (141). Wildtype E. coli cells can survive treatments which produce 55-70 crosslinks per genome, and recombination defective (recA), or excision defective (uvrA) strains, survive after the introduction of 5-20 crosslinks per genome (34, 35). However, a double mutant deficient in both excision-repair and recombinational-repair (recA, uvrA) is killed by treatments producing a single crosslink per genome (34). This indicates that interstrand crosslinks are a repairable lesion both in animal systems and in bacterial cells. The response of repair deficient mutants of E. coli to psoralen-plus-light suggests that both excision-repair and recombinational-repair steps are required to complete the

repair of an interstrand crosslink (142).

The scheme in Figure 4 shows a possible mechanism for the repair of interstrand crosslinks in DNA (142). A similar scheme has been presented for the post-replication repair of UV-induced dimers (132). The obvious similarity of interstrand crosslinks and post-replication gaps containing pyrimidine dimers is that these kinds of structural damage to the DNA result in damage to both strands of the duplex in close proximity. This results in a defective sequence of bases adjacent to complimentary DNA which is similarly damaged (i.e., covalent crosslink), or a defective sequence with no complimentary DNA to use as a repair template (i.e., pyrimidine dimer in a gap). Therefore, it is very likely that the repair of post-replication gaps, and covalent interstrand crosslinks involves the utilization of similar repair pathways and intermediate structures.

Steps 1-6 in Figure 4 show the proposed intermediate structures and repair processes which occur during the repair of covalent interstrand crosslinks in DNA (142). The damage shown in step 1 (Figure 4) is first recognized by $\underline{uvrA}^+$ endonuclease, which introduces incision breaks in one strand of the crosslinked duplex, on both sides of the crosslink arm. Following this excision-repair step, the DNA assumes the configuration shown in step 2 and can enter the recombinational-repair pathway in strains with functional $\underline{recA}^+$ gene product. Entry into the $\underline{rec}$ repair

Figure 4.--The repair of interstrand crosslinks.
One possible mechanism for the repair of crosslinked DNA
is shown here to illustrate the types of products and
intermediates involved, and to indicate steps controlled
by the uvr and recA genes. The sequence proceeds as
follows: Two incisions are first made near each crosslink.
A nuclease then widens the gap, exposing a single-stranded
region. Ensuing strand exchanges between homologous
duplexes insert an intact base sequence complementary to
the strand still carrying the partially excised cross-
linking residue. When the twin helical DNA structure is
restored, the remaining arm of the crosslink is excised.
Dashed lines indicate regions of repair synthesis (142).

The Repair of Interstrand Crosslinks

1. homologous duplexes

   ↓ excision (uvrA)

2.

   ↓ strand exchanges (recA)

3.

   ↓ branch migration and repair synthesis

4.

   ↓ duplex separation

5.

   ↓ excision and repair synthesis

6. intact duplexes
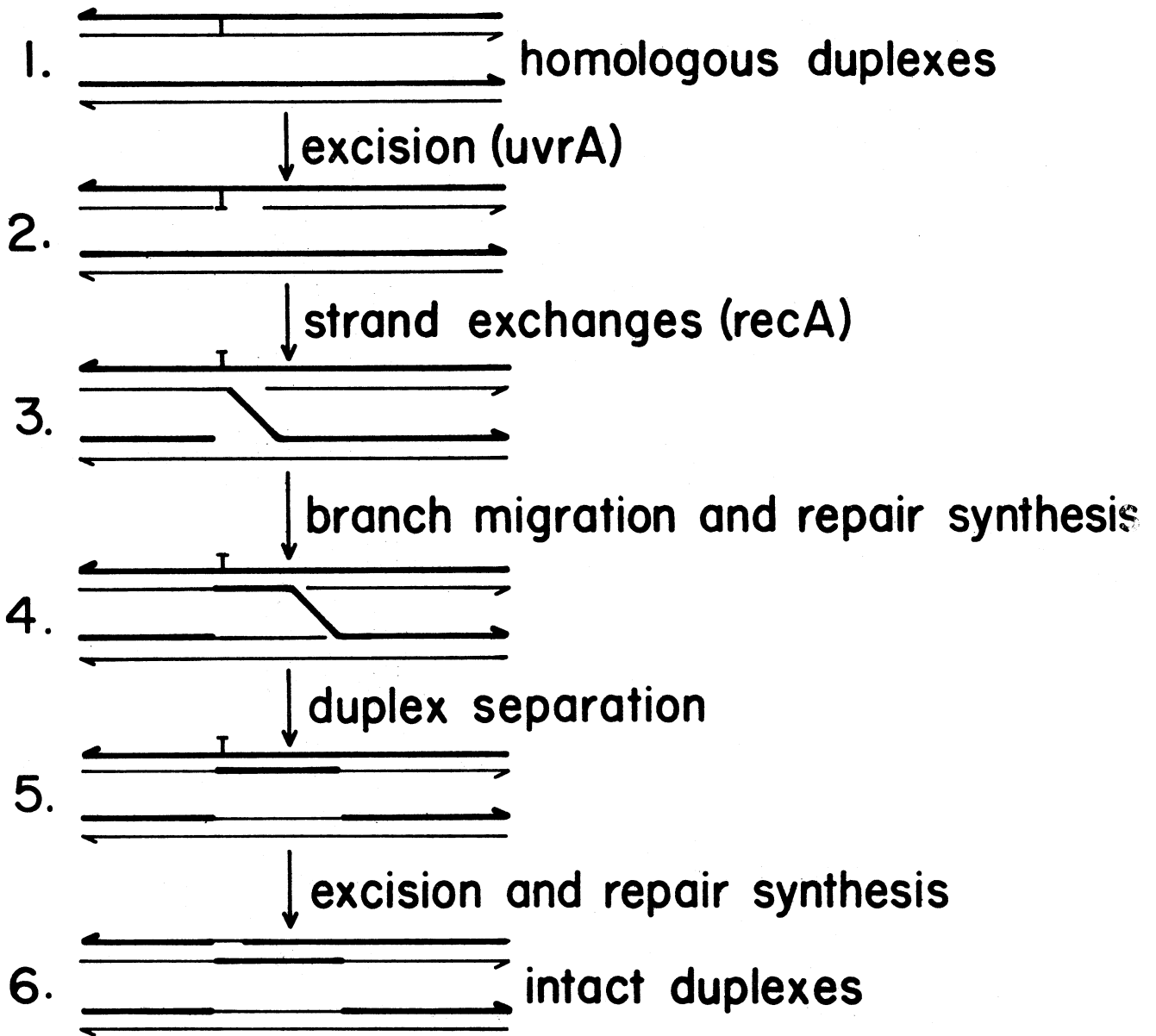
Figure 4

pathway results in strand exchanges; this produces the intermediate configurations shown in steps 3 and 4. The repair synthesis shown in step 4 during the process of branch migration may be a function shared (or carried out exclusively) by DNA polymerase III (polC). Following the insertion of an undamaged template from homologous duplex DNA, separation of the two recombinant molecules takes place as shown in step 5. The repair of the DNA crosslink is then completed by the excision-repair of the remaining crosslink arm using the recombinant DNA segment as a template for repair synthesis (step 6).

An interesting problem with this proposed scheme is that it would appear at first glance that both excision-repair and recombination repair are required to success-fully repair a single DNA crosslink. This is not con-sistent with the observation that uvrA or recA mutants can repair 5-20 crosslinks per genome while recA uvrA double mutants are killed by only 1 crosslink. However, recent evidence indicates that psoralen-DNA monoadducts can persist in the DNA for long periods without significant disturbance of DNA synthesis rates or reduction in cell viability (Cole, R. C., personal communication). Therefore, the excision of the crosslink in a recA mutant might allow the successful replication of DNA containing some partially removed crosslinks. This would account for the observation that recA mutants can repair a few crosslinks without the

ability to initiate (and/or complete) the strand exchange
step of crosslink repair. The repair which occurs in
uvrA mutants is probably the result of non-specific
incision breaks made by other endonucleases in the cell
resulting in the probable repair of crosslinks to step 5.
The uvrA strain could then "dilute-out" the damage by
subsequent replication of the DNA containing the crosslink
copy. If this process introduces new "post-replication
gaps" then the recombination repair system can replace
the information at each new round until the dilution
process has eliminated the presence of the lesion from
any significant fraction of the population. In fact,
recent work on post-replication repair of UV-induced
dimers in uvrA strains in vitro, suggests that the
recombination repair of post-replication gaps with dimers
results in the even distribution of the dimers in parental
and daughter DNA (143, 144). Therefore, post-replication
repair and recombinational-repair of interstrand crosslinks
probably involves the utilization of both the rec repair
pathway and the excision-repair pathway (132, 142). This
concept of "coordinated recombination-excision repair"
is an important innovation in understanding the function
of DNA repair in normal cells. An important question comes
into focus: What processes and/or gene products regulate
the interaction between excision and recombinational repair
pathways?

DNA repair functions evolved very early and were retained throughout evolutionary time. This is demonstrated graphically by the recent observation that human leukocytes can photoreactivate 254 mm UV-induced damage (145). Since photoreversal of dimers is a very specific enzymatic reaction, this is good preliminary evidence that mammalian cells have a repair enzyme with a function similar to a bacterial enzyme, i.e., the photoreversal of cyclobutyl pyrimidine dimers in DNA. A variety of animal cells have been shown to be capable of excision repair processes (146-148), and a genetic deficiency of human cells in excision repair has been associated with the incidence of skin cancer in the disease Xeroderma pigmentosum (149). Post-replication repair has also been demonstrated for animal cells. However, the gaps may be filled without any strand exchange (150-155). Thus, a great deal of evidence now indicates that DNA repair processes occur throughout the biological world, and play an important role in DNA metabolism.

B. PHOTOBIOLOGY OF FAR AND NEAR-UV RADIATION

1. Introduction

The electromagnetic spectrum includes the entire range of wavelengths of electromagnetic radiation from gamma rays to long wave radio signals including ultraviolet and visible light. While there are striking differences

in the biological and chemical response to irradiation
with energy from the various regions of the electromagnetic
spectrum, it should be noted that the physical charac-
teristics of electromagnetic energy are basically the
same. All of the energy in the electromagnetic spectrum
is packaged in precise quanta called photons which move
at the speed of light in a vacuum (3 x 10^{10} cm/sec). The
energy of these photons is inversely related to their
wavelength according to the following simple equation:
$E = \dfrac{12,400}{\lambda}$ where λ equals the wavelength in Angstroms and
E equals energy of the photon in electron volts (156).
Thus, the only physical difference between various wave-
lengths of electromagnetic energy is the wavelength thru
which the photons travel and the amount of energy packaged
in each photon.

The striking differences in the biological and
chemical action of various wavelengths are the kind of
interactions which photons of various energies have with
atoms and molecules. The scheme in Figure 5 shows the
wavelength of photons of various energies in the electro-
magnetic spectrum, the photon energies in electron volts,
the bond energies of chemical bonds with which each range
can interact, and the photochemical result of molecular
absorption of photons in each energy range (157). When
absorption of a photon more energetic than ca. 13 electron
volts occurs molecular or atomic ionization is a likely

Figure 5.--The electromagnetic spectrum (157).

THE ELECTROMAGNETIC SPECTRUM

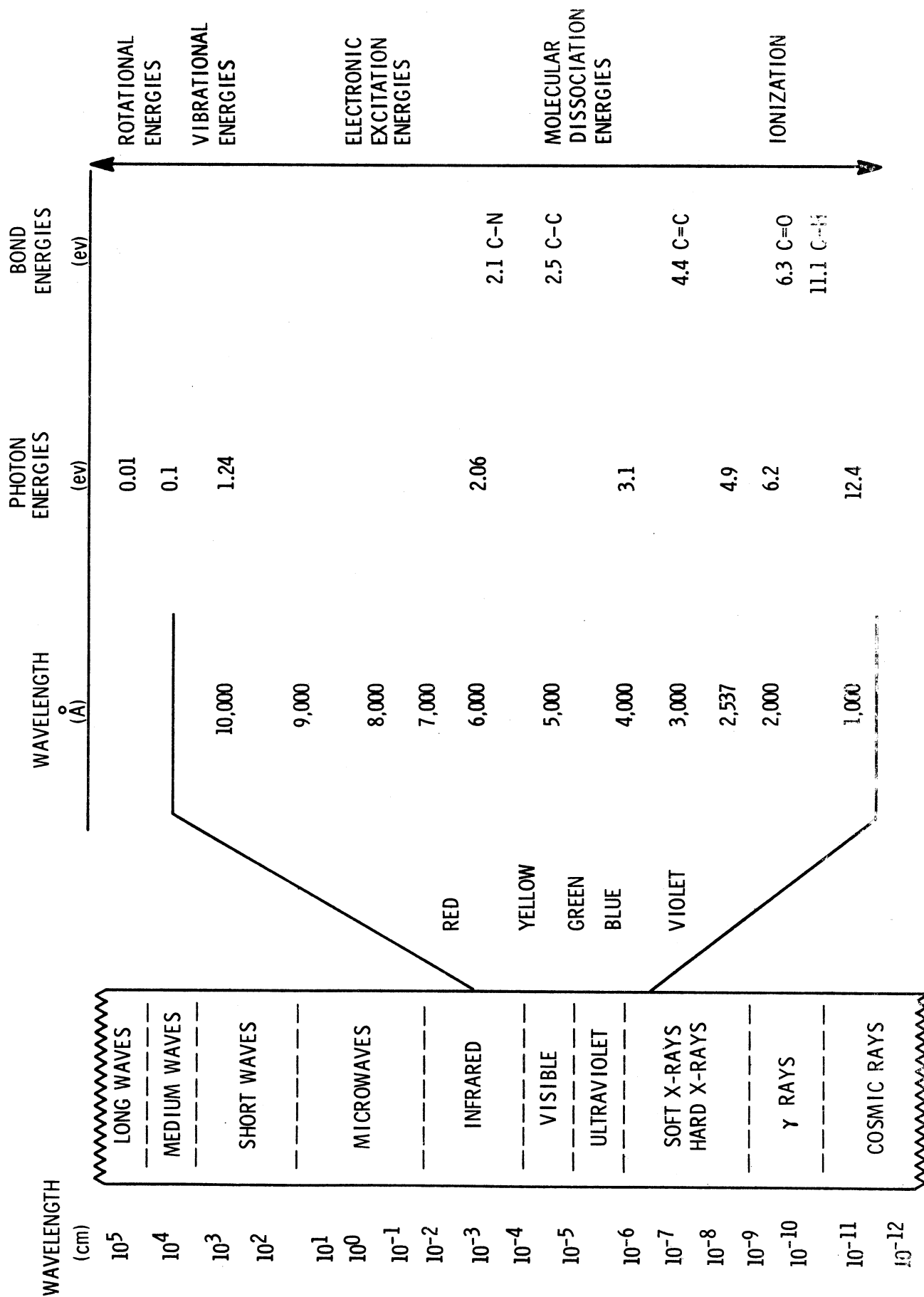| WAVELENGTH (cm)		WAVELENGTH (Å)	PHOTON ENERGIES (ev)	BOND ENERGIES (ev)	
10^5	LONG WAVES		0.01		ROTATIONAL ENERGIES
10^4	MEDIUM WAVES		0.1		VIBRATIONAL ENERGIES
10^3	SHORT WAVES	10,000	1.24		
10^2					
10^1	MICROWAVES	9,000			
10^0		8,000			ELECTRONIC EXCITATION ENERGIES
10^{-1}		7,000 RED			
10^{-2}					
10^{-3}	INFRARED	6,000	2.06	2.1 C–N	
10^{-4}					MOLECULAR DISSOCIATION ENERGIES
10^{-5}	VISIBLE — YELLOW, GREEN, BLUE	5,000	3.1	2.5 C–C	
10^{-6}	ULTRAVIOLET	4,000 VIOLET	4.9		
10^{-7}	SOFT X-RAYS / HARD X-RAYS	3,000		4.4 C=C	
10^{-8}		2,537			
10^{-9}	γ RAYS	2,000	6.2	6.3 C=O	IONIZATION
10^{-10}				11.1 C–H	
10^{-11}	COSMIC RAYS	1,000	12.4		
10^{-12}					

Figure 5

photochemical event. The absorption of these relatively high energy photons results in the ejection of an electron of elevated energy which can interact with a number of other molecules or atoms nearby. Thus, the absorption of a small number of high energy photons can result in the cascading of a great number of photochemical events due to secondary the effects of each absorption. The interaction of ionizing irradiation with one molecule can affect the chemical state of many neighboring molecules and thereby magnify the net effect of the irradiation. As we go down the photon energy scale to visible and ultraviolet wavelengths molecular dissociation is the primary photochemical result of absorption. Since secondary "chain" events do not result from absorption of less energetic photons, and given absorption produces a net of only a single photo-chemical change.

The first law of photochemistry (Grotthus-Draper Law) states that light must be absorbed by a molecule before photochemistry can occur. Therefore, energy which passes thru a molecule without being absorbed can do no photochemical work on the molecule. This law is very important in understanding the difference between the photobiology of irradiation with high energy photons (e.g., X-rays) and relatively low energy photons (UV or near-UV). Conceptually, a photon absorption is somewhat like a magician's hat trick. What he places in the hat

comes out changed. When a molecule absorbs a photon it must change. The exact nature of this change is a function of the amount of energy absorbed and the chemical and electrical state of the molecule in question. Herein lies another important difference between the photochemical characteristics of high energy and low energy photons. While absorption of photons of less than 3.1 electron volts is a function of molecular structure (absorption spectra), high energy photons are absorbed as a function of electron density of the sample irradiated. Therefore, differences in molecular structure within the same relative electron density range make little or no change in the amount of gamma or X-ray energy absorbed. Thus, ionizing irradiation differs from non-ionizing irradiation in the criteria for molecular absorption of the energy, and the kind of photochemical interaction which occurs between the absorbed photon and the molecule or atom which absorbs the energy.

2. Dosimetry

Techniques for the measurement of radiation dose may be divided into three categories. Physical techniques measure radiant energy by measuring some physical change effected by the irradiation. The most fundamental device for measurements in the ultraviolet region of the spectrum is the thermopile, which absorbs radiant energy and converts it to heat which in turn alters the electrical

potential across the junction between two dissimilar
metals. A similar device which has the advantage of being
cheaper and less fragile than the thermopile is the
radiometer (i.e., YSI Kettering). This device provides
the convenience of a physical technique and can be
accurately calibrated with a thermopile. Also, a thermopile
should be calibrated with a 50 watt carbon filament standard
lamp (National Bureau of Standards) approximately once
every year.

Chemical techniques, which measure the chemical
change produced by the radiation, are called "actinometry".
Actinometry by means of liquid solutions is the best
technique for determining the total dose of radiation
when (a) the sample has an unusual geometry or a geometry
that varies with time, (b) the radiation field is non-
uniform, or (c) the dose rate fluctuates during the experi-
ment. The use of potassium ferrioxalate solutions (158)
for actinometry is reviewed in Introduction to Research in
Ultraviolet Photobiology (159) and by McLaren and Shugar
(160).

Biological techniques are sometimes useful.
However, this is generally restricted to inactivation of
viruses, and care should be taken to use the same host,
incubation conditions, etc. Biological techniques are
also important for the evaluation of dose rate effects with
high energy sources, and to test for different biological

effects with different "kinds" of light, e.g., coherent
light from lasers. However, the maximum reproducability
from experiment to experiment for physical techniques
make these techniques the most generally used in photo-
biology. The measurement of energy in the UV and near-UV
region of the spectrum can be accurately measured (for
dose rates > 5 erg/mm^2/sec) with a radiometer.

Radiometry is a generally applied terminology
which uses the c.g.s. system of units and applies to all
electromagnetic radiation. The term radiant energy
(erg, or calorie) relates to the beam of radiation passing
through space without regard for its origin or destination.
Radiant flux (watt/erg/sec), refers to the power of the
beam, and radiant flux density (watt/cm^2) is the power
crossing a unit area normal to the beam. Terms of
radiometry which describe the source of radiation are
radiant intensity (watt/steradians), which is the power
emitted per solid angle into space by the entire source,
and radiance (watt/steradian/cm^2) which relates the power
emitted per solid angle into space by a unit projected
area of the source. The terminology of dosimetry is
described by terms relating to the object struck by the
radiation. Irradiance (watt/cm^2, erg/cm^2/sec) refers to
the power striking the object per unit area of the object,
or energy per unit area per unit time. In biological
applications, especially for bacterial work, irradiance

is expressed in erg/mm^2/sec. However, in an effort to

make bacterial dosimetry more "relevant" to include solar

radiation studies, many journals currently require that

irradiance be expressed in joules/meter^2/sec. This is

conceptually very awkward since one never irradiates a

meter^2 cuvette. Therefore, I will frequently discuss

dosimetry in erg/mm^2/sec even though figure legends will

always be expressed in J/m^2/sec. The conversion of these

units is as follows: 1 J/m^2/sec = 10 erg/mm^2/sec.

Dosimetry is a terminology which applies only to

biological objects struck by the radiation. The terminology

applied here will use radiometric terminology exclusively

(photometry is a parallel terminology related to human

vision), since the energy discussed here is not visible to

the normal human eye. The integral dose (ergs/cell) is

the total radiant energy striking the object. The dose

rate (ergs/mm^2/sec) is analogous to irradiance and

describes the power striking the object per unit area.

When considering irradiation of a biological object with

non-ionizing energy, dose is described in terms of "incident

energy", or energy which strikes an object. This does

not imply that the energy is absorbed, in fact, when

absorption of any meaningful fraction of the energy

occurs the "average incident dose" must be calculated to

account for the missing energy. The following equation

will be used to calculate the average incident dose of

samples irradiated in solutions which absorb more than

10% of the incident energy: $(I_O - I)$ $1/ \ln \frac{I_O}{I} = \gamma$ where I_O

equals the amount of radiant energy at the front of the

sample, and I equals the amount of energy which passes

through a 1 cm sample (usually calculated from %T on a

spectrophotometer), and γ equals the "average incident

dose" (or dose rate) for a given sample.

In contrast to photobiological dosimetry, radio-

biology (biological studies with ionizing radiation)

uses absorbed dose (called exposure) to measure the amount

of radiant energy delivered to any given sample. The

usefulness of absorbed dose for measuring ionizing

radiation is dependent on the fact that any given sample

of tissue, bone, or water has a finite probability for

the absorption of energy as a function of the electron

density of the sample. The basic unit of dosimetry for

ionizing radiation is the rad. Exposure of a sample to 1

rad of X-ray energy corresponds to the absorption of

100 ergs/g of sample material irradiated. All of the

dosimetry of ionizing radiation presented here will be

expressed in terms of the rad.

3. The Photobiology of Far-UV Radiation

For this discussion, far-UV describes the region

of the spectrum between 240 nm and 280 nm, and will

generally apply to irradiation with a germicidal low

pressure mercury light source which emits a large amount

of 254 nm energy (161). There are virtually thousands of publications about the photochemical and photobiological effects of far-UV irradiation. Therefore, no attempt will be made to comprehensively review this section of the literature.

3.1 Far-UV Inactivation and Mutation.

There is a preponderance of evidence which indicates that absorption by DNA is responsible for the major portion of the effects on cells produced by far-UV. This applies especially to killing and mutation. The action spectra for killing which Gates measured in 1930 for E. coli and Staphylococcus aureus corresponded more closely to the absorption spectrum of nucleic acids than protein (162). Action spectra of inactivation and mutation conducted by other workers verified this and raised the question as to whether or not the DNA was indeed the target molecule (163-165). Also, the initial effect of far-UV irradiation is probably purely photochemical since there is little or no temperature coefficient for these effects (165). The absence of a temperature coefficient for far-UV irradiation indicates that enzymatic interaction or organic reactions with the photon is not a requirement for the primary effects of the irradiation. Photochemical reactions are to a large extent not influenced by temperature (with the exception of differences over wide ranges). There are several review articles which present extensive evidence

that the DNA molecule is the principle target molecule

(at least in simple systems) for far-UV irradiation (7,

166-168).

However, probably the most important observation

about far-UV effects was the detection of a particular

lesion in the DNA as a result of far-UV irradiation of

DNA in vitro. While searching for photoproducts in

solutions of DNA irradiated with far-UV light sources,

the dimerization of adjacent pyrimidine residues was

found to be a major photoproduct (169, 170). There are,

of course, other types of lesions in the DNA as a result

of far-UV irradiation (166, 167, 171), which may account

for part of the lethal effects especially in repair

sufficient strains. However, pyrimidine dimers really

represent the cornerstone in the argument for DNA repair

and DNA as the target molecule. The dimer is the best

documented lesion both as a repair lesion and as a

change in the DNA which is a radiation induced lesion (171).

The formation of cyclobutyl pyrimidine dimers is

a photochemically reversible reaction which can be kineti-

cally described as follows:

$$-PyPy- \underset{k_r}{\overset{k_f}{\rightleftarrows}} -PyPy-$$
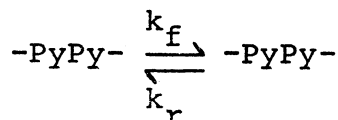
Similar photoreversibility is not observed for the "other"

far-UV lesions, such as intrastrand crosslinks,

DNA-protein links, or hydrate formation. The forward
and reverse constants for the dimer reaction are dependent
on wavelength and experimental conditions (172). There-
fore, the photoreversibility can be used to correlate
the biochemical presence of pyrimidine dimers with
biological effects which result from the presence of
dimers in the DNA. By similar logic, if photobiologic
reversibility of far-UV effects requires the same condi-
tions that photochemical reversal of dimers requires, then
this can be taken as evidence dimers have a biological
effect.

The detailed photochemistry of pyrimidine dimers,
first discovered as photoproducts of thymine irradiated
in aqueous solution (169), has been the subject of
numerous studies on model polynucleotides and oligo-
nucleotides (16, 161, 173-177). These studies of a
purely photochemical event were of great importance in
determining the role of dimers in the biological effect
of far-UV because they established the physical conditions
for photoreversal and characterized the chemistry of
dimers. Whole biological systems (even phage particles)
are very sensitive to the short wavelengths which are
used for direct photoreversal experiments. However,
Hemophilus influenzae transforming DNA, which is in-
activated with 280 nm, can be directly photoreactivated
by subsequent treatment with 239 nm radiation (15, 178).

The kinetics of this reaction are quantitatively similar to those for dimer monomerization, and quantitative analysis of high doses indicates that 50 to 70% of the biological inactivation may be attributed to pyrimidine dimers in the DNA.

Probably the strongest evidence for the role of far-UV dimers in mutation and cell inactivation comes from studies of enzymatic photoreactivation. The work on photoreactivation has been extensively reviewed (179). Very early work of Dulbecco showed that UV-inactivated phage were only photoreactivable after they had infected their hosts (18, 180). When later work clearly showed that phage infection could only occur after the virus DNA has penetrated the cell (181), this finding suggested that cellular processes were involved in the photoreactivation of phage. This conclusion was supported by the observation that the action spectra of host and virus photoreactivation were very similar (182). The photo-reversibility of far-UV induced mutations further implicated the DNA in this process (183, 184).

The most convincing evidence that photoreactivation by exposure of cells to wavelengths between 350 and 450 nm after inactivation with far-UV is an enzymatic process comes from in vitro studies with extracted photoreactivating enzyme (PRE) (185, 186). This in vitro photoreactivation system provides a means to test the PR enzyme on irradiated

polynucleotides by observing the competition of these chemically defined molecules for PRE activity on transforming DNA (187). The ability of PR enzyme and photoreactivating light to act on particular photoproducts in far-UV irradiated polynucleotides can be assessed by the competition of these chemically defined photoproducts for in vitro reactivation of irradiated transforming DNA. Polymers which compete most efficiently for the binding of PR-enzyme with irradiated transforming DNA are irradiated polynucleotides which contain adjacent pyrimidine residues (188). This competition may be reversed by pretreating the irradiated polynucleotide with PRE plus light. However, far-UV irradiated RNA does not compete with the in vitro photoreactivation of transforming DNA (189). Therefore, pyrimidine dimers in RNA probably do not interfere with the reactivation of DNA by PRE and light in vivo.

A correlation between the presence of PRE in cell extracts, and in vivo photorepair of cellular DNA, with the recovery of colony forming ability of irradiated cells, indicates that PRE is responsible for most of the in vivo photorecovery observed (190). The genetic evidence for the involvement of PRE in photorecovery substantiates this correlation, and leaves little doubt about the importance of PRE to cellular photorecovery. The isolation of a photoreactivation deficient mutant

(phr) of E. coli B which lacks both PRE activity in cell extracts and the ability to demonstrate cellular photo-recovery of far-UV damage, provides genetic evidence for the role of PRE in vivo (191). Thus, the work on PR-enzyme provides the strongest case for the involvement of a radiation induced lesion in the DNA of the cell in far-UV cell inactivation and mutation.

The dark repair of far-UV damage was discussed in detail in the DNA repair section of this review. However, a note of caution must be presented about the over zealous interpretation of pyrimidine dimers as lethal and mutagenic lesions in the DNA. A great deal of the evidence which supports this view has been obtained in tests on repair deficient strains of bacteria. Therefore, one can un-equivocally conclude that most of the far-UV damage which causes the inactivation of repair deficient strains, and virtually all of far-UV mutation of these strains, results from the induction of dimers in the DNA of the cell. The far-UV inactivation and mutation of wildtype strains of bacteria which can repair many dimers may require the presence of some additional "other" lesion in the DNA for the dimers to be biologically lethal. There is some indirect evidence for this. A simple catalogue of DNA lesions present following a D_{37} dose (mean lethal dose) of far-UV for E. coli B shows that the DNA contains 800 pyrimidine dimers, 1.3 interstrand cross-

links, 0.4 single strand breaks, and 0.2 protein-DNA

links (171). Since single strand breaks and protein-DNA

links occur less frequently than once per mean lethal

dose for this strain it can be argued that these lesions

are not important to far-UV inactivation. However, the

presence of 1.3 interstrand crosslinks per mean lethal

dose allows the argument that the 800 dimers might not be

lethal if the crosslink were not present in the DNA.

Thus, while the pyrimidine dimer is certainly the most

important DNA lesion for far-UV irradiation of biological

systems, care should be taken to separate dogma from fact.

4. The Photobiology of Near-UV Radiation

In this discussion the term near-UV refers to

electromagnetic energy between 310 nm and 450 nm.

Monochromatic near-UV will refer to irradiation of samples

with light emitted from a monochromator with cutoff

filters imposed to reduce scattered light, and broad

spectrum near-UV effects refer to irradiation with a low

intensity filtered BLB light source emitting energy between

310 nm and 450 nm. This broad spectrum source has a

maximal output at 365 nm and the detailed emission spectrum

is shown in Figure 6 (personal communication R. B. Webb).

4.1 Introduction.

Near-UV and visible light was first reported to

be lethal for bacteria by Blum in 1941 (192). A short time

later Hollaender demonstrated in quantitative terms that

Figure 6.--The emission spectrum of a broad spectrum near-UV light source (BLB blacklight) (R. B. Webb personal communication).
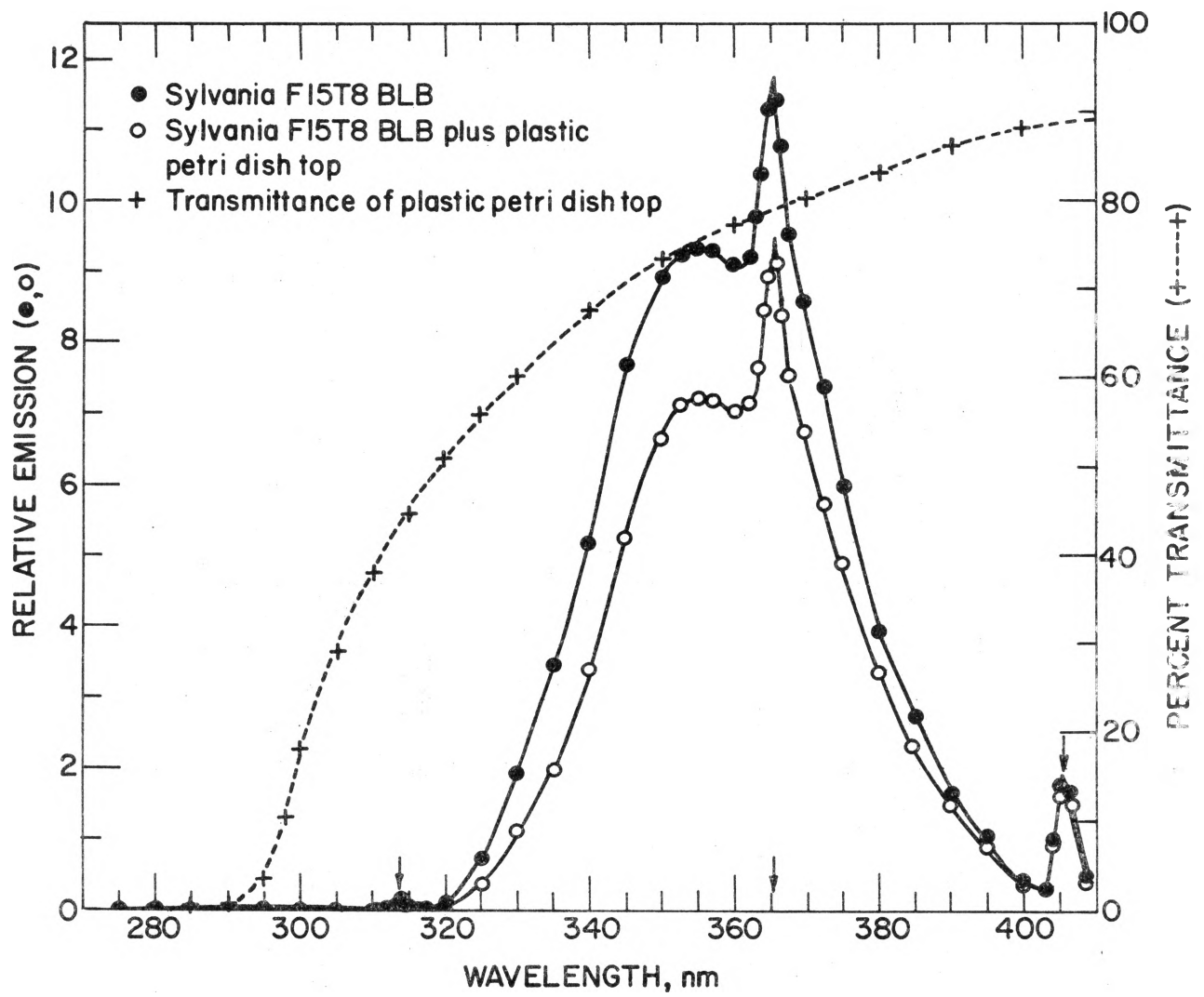
Figure 6

wavelengths longer than "germicidal" far-UV were lethal for bacteria (193). However, these reports and many to follow were generally attributed to "leaky" effects of trace amounts of far-UV present in the scattered light emitted by the radiation source. The imposition of various combinations of glass and chemical filters during long wavelength experiments have really eliminated this argument for the monochromatic light experiments, and recent advancements in phototechnology have resulted in the availability of equipment to accurately measure the amount of energy at each wavelength of the emission spectrum of broad spectrum sources. This improved instrumentation indicates that BLB blacklight emits virtually no energy of wavelength shorter than 310 nm (Figure 6). The important feature of the instrumentation for broad spectrum work is that synergistic effects of wavelengths within the near UV-region can now be studied with reasonably accurate dosimetry.

4.2 Near-UV Mutagenic Effects.

Hollaender and Emmons were the first to report that wavelengths longer than 290 nm are mutagenic for spores of Aspergillus terreus and other fungi (194). This report included the finding that sunlight is mutagenic, and attributed most of this mutagenicity to wavelengths between 290 nm and 320 nm (194). Kaplan later demonstrated that energy between 313 nm and 380 nm caused the mutagenic

induction of the white variant of Serratia marcescens (195).

Kubitschek found that a WP2 hcr strain of E. coli is

mutated to T_5 resistance by irradiation with 330 nm to

380 nm light (196). The mutation of WP2 hcr to T5

resistance occurs by a mechanism which probably does not

involve pyrimidine dimers (196). The mutagenicity of

chronic irradiation with low intensity broad-spectrum

near-UV for chemostat cultures of E. coli B/r and B/r/1 t⁻

to T5 resistance (197, 198) was shown to occur via an

oxygen dependent process for wavelengths between 340 nm

and 500 nm (198). This work included an action spectrum

for the induction of T5 resistant mutants by chronic

exposure to energy between 300 nm and 600 nm. The action

spectrum indicated that oxygen dependent mutagenesis peaked

at approximately 450 nm with a minimum of action at 350 nm

and 550 nm (198). The importance of oxygen dependence

for near-UV mutation is the indication that direct

absorption of energy by DNA does not account for the

observed mutagenicity. Experiments which involve long

term exposures to near-UV at room temperature almost

certainly do not involve pyrimidine dimers since this

treatment would be expected to photoreactivate any dimers

which might appear during the irradiation.

Monochromatic 365 nm near-UV mutagenesis which

occurs as the result of acute irradiation is almost

certainly caused by pyrimidine dimers in the DNA. Acute

exposure of E. coli WP2 hcr trp to monochromatic 365 nm
energy increases the number of tryptophan revertants
substantially (R. B. Webb, unpublished results). This
365 nm mutagenic effect is oxygen independent and approxi-
mately 90% of the premutational lesions are photo-
reactivable. Therefore, the monochromatic 365 nm pyrimidine
dimer (199) is an oxygen independent lesion in the DNA
which probably accounts for nearly all of 365 nm mutagen-
icity. However, since broad spectrum near-UV mutagenesis
is strongly oxygen dependent (198) and monochromatic
365 nm light induction of pyrimidine dimers is oxygen
independent it is likely that a DNA lesion other than the
dimer is responsible for broad spectrum near-UV mutagen-
icity. Monochromatic 365 nm mutagenesis (primarily due
to dimers) may result from the direct absorption of this
energy by the DNA, or via a sensitized process which does
not involve oxygen.

With the exception of acute exposure of cells to
monochromatic 365 nm light, little in the way of factual
information is known about either the chromophore or the
DNA lesion responsible for near-UV mutagenesis. Action
spectrum information is consistent with riboflavin or
porphyrins as the mutagenic chromophore (198). However,
it has not been established that any of the suggested
candidates for the chromophore role can sensitize the
mutational process when added to the medium in which cells

are irradiated (R. B. Webb, personal communication).
The recent observation that photoproducts of L-tryptophan
synergize the production of T5 resistant mutants in
chemostat cultures of E. coli B/r/1 t⁻ irradiated with
broad spectrum near UV may reopen the possibility of
detecting a near-UV mutational lesion in the DNA. Recent
work with photoproducts of L-tryptophan (TP) have shown
that TP increases the rate of induction of "alkali-labile
bonds" in bacterial DNA, when cells are irradiated with
monochromatic 365 nm light in solutions containing TP
(Yoakum, manuscript in preparation). Synergism of TP and
365 nm irradiation for the inactivation of E. coli p3478
polA1 indicates that while the TP sensitized DNA lesions
do contribute to the inactivation of this strain, a large
number of these "alkali-labile bonds" are repairable.
Future experiments are planned to test broad spectrum
near-UV and TP for the synergistic production of DNA
lesions.

    4.3 Near-UV Lethality.

    There have been numerous reports on the harmful
effects of near-UV irradiation of biological systems
without external sensitizers present during the exposure
(192, 200-202). The growth phase of cells at the time
of near-UV irradiation is a strong modifier of near-UV
lethality. In contrast to far-UV inactivation, irradiation
of repair deficient strains with near-UV is much more lethal

if the cells are in the log phase of growth (203). This
log phase sensitivity to near-UV is especially notable for
recombination deficient (rec) mutants exposed to broad
spectrum near-UV (204, 205). Unfortunately, the broad
spectrum light sources used in these studies do not pro-
vide enough radiant intensity to kill wildtype cells.
Therefore, interpretation of the log phase sensitivity of
these strains is difficult.

Experiments conducted with natural sunlight have
shown that wavelengths in the solar spectrum are lethal
for a wide range of microorganisms. Sunlight has been
observed to inactivate bacteriophage, TMV RNA, and
transforming DNA. In addition sunlight damage can be
modified by DNA repair processes (206, 207). Much of the
lethal action of sunlight has been attributed to the
shorter wavelengths in the spectrum (310 nm). However,
the discovery of near-UV synergistic effects may clarify
the role which longer wavelength near-UV light plays in
cell inactivation.

Monochromatic near-UV experiments include a great
deal of work with 365 nm wavelength energy. Monochromatic
365 nm inactivation of repair deficient and wildtype
E. coli strains is strongly oxygen dependent (208).
However, an important exception to this is the observa-
tion that E. coli AB2480 recA uvrA is not strongly oxygen
dependent for monochromatic 365 nm light inactivation

(Webb, personal communication). Since the induction of
pyrimidine dimers is not oxygen dependent and this strain
shows a significant photoreactivable sector following
365 nm inactivation (209), it is very likely that nearly
all of the monochromatic 365 nm lethality observed for
AB2480 is the result of dimers in the DNA. Monochromatic
365 nm inactivation of wildtype, rec, or uvr strains
probably involves a non-dimer DNA lesion. This is based
on several observations: a) 365 nm inactivation for these
strains is oxygen dependent and dimer formation is not,
b) recA strains show photoprotection to 254 nm inactiva-
tion when they are preirradiated with 365 nm light, c)
recA, uvrA, and wildtype E. coli do not show a significant
photoreactivation sector following 365 nm inactivation.
It is very likely that some non-dimer DNA lesion which is
repairable accounts for the lethality of monochromatic
365 nm irradiation for rec, uvr, and wildtype E. coli.

Many of the effects of broad spectrum near-UV
have been attributed to the action of wavelengths between
260 nm and 320 nm. Recent improvement in our ability to
accurately determine the exact energy emitted by various
broad spectrum light sources have shown that a broad
spectrum near-UV light source (filtered BLB blacklight)
emits less than 0.2% 313 nm energy and virtually no
energy below 310 nm (Figure 6). The error in previously
published spectra for broad spectrum near-UV light sources

was the result of a failure to filter out long wavelength scattered light in the peak of the spectral sensitivity of the phototubes used to measure radiant energy (R. B. Webb, personal communication). This means that most of the broad spectrum near-UV effects may be synergistic action of wavelengths within the near-UV region. If this near-UV synergism is a central near-UV effect then broad spectrum near-UV experiments may involve completely different lesions and mechanisms of action from those observed for monochromatic wavelengths within the near-UV region of the spectrum.

There is room for a great deal of speculation about possible near-UV chromophores, targets, and lesions responsible for biological inactivation. However, it is now quite evident that many cell processes (i.e., DNA repair) (210) are affected by near-UV energy and it is not likely that any single chromophore or lesion is totally responsible for near-UV lethality. Some significance may be placed on the recent discovery of an oxygen dependent DNA lesion induced by 365 nm irradiation. That is, 365 nm induced alkali-labile bonds (211) which are strongly oxygen dependent may play a major role in 365 nm monochromatic light inactivation. However, a great deal more work must be done in the area of near-UV DNA lesions before the role of these lesions in cell inactivation can be accurately interpreted.

III. MATERIALS AND METHODS

A. THE EFFECT OF TP ON DNA REPLICATION
AND CELL DIVISION

1. Bacterial Strains

The following strains were used in the experiments
described below: W3110 wildtype, thy; AB2500 uvrA, thy;
P3478 polA1, thy; X9247 recA recB thy. All strains used
were derived from Escherichia coli K12. The genotypes
and phenotypes are listed in Table 1.

2. Growth and Media

Log phase cells were obtained for labeling and
growth experiments by 1/20 dilution of overnight cultures
which had grown through at least 20 generations of log
phase growth, and had not exceeded 2×10^8 cells/ml.
Minimal medium was buffered with M9 salts as follows
(μg/ml): glucose, 400; L-tryptophan, 20; L-proline, 10;
L-histidine, 10; casamino acids (vitamin free), 200; niacin,
1; thiamine, 1; and thymine, 10. The M9 salts contained
the following in g/liter: NH_4Cl, 1.0; $Na_2HPO_4 \cdot 7H_2O$, 6.0;
KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 0.2; and NaCl, 0.5. This medium
was used for growth in all experiments and will hereafter
be referred to as M9 medium. The cells were incubated in
a 37^OC constant temperature room, and all experimental

Table 1. List of E. coli strains described in the text

Strain No.	Genetic Markers	X-ray Response (131)	UV Response (59)	TP* Response % Survival
W3110	nic, thy	resistant	resistant	94
P3478	nic, thy, polA1	sensitive	moderately sensitive	89
AB2500	thi, thy, arg, pro his, leu, uvrA	resistant	sensitive	91
X9247	thy, recA, recB	sensitive	sensitive	5

*TP sensitivity was measured by incubation of log phase cells in TP-M9 medium (equivalency of 150 µg/ml unirradiated tryptophan) for 30 min with subsequent dilution and plating on nutrient agar plates which were incubated 48 hr to determine number of survivors. Greater than 85% survival indicated resistance, less than 10% survival indicated sensitivity. Since log phase rec cells are most sensitive to TP in the first cycle of killing, and percent survival below 1-10% is not exponentially reduced, only the first cycle of killing was used to determine toxicity (212, 213).

manipulations were carried out in this room with prewarmed materials and media.

3. Photoproduct Preparation

Tryptophan photoproduct was prepared by aerobic irradiation of a 5 mg/ml aqueous solution of L-tryptophan (pH 8.2) to an approximate absorbed dose of 1×10^7 erg/mm^2 of 290 nm light from a Honovia 2.5 kW xenon mercury arc lamp and a Baush and Lomb 500 mm monochromator. The monochromatic light included some long wavelength scattered light and an approximate dispersion band of 280-300 nm. This light source was chosen for use following an action spectrum study which indicated that 290 nm is the most effective wavelength for the production of tryptophan photoproduct (212). The results of the action spectrum for the production of toxic photoproducts of L-tryptophan are shown in Figure 7 (212). However, it is also possible to make a similar toxic photoproduct of L-tryptophan with a broad spectrum blacklight (213). The tryptophan photoproduct (hereafter referred to as TP) was diluted into M9 medium (referred to as TP-M9) to a final concentration equivalent to 375 μg/ml of tryptophan in the unirradiated solution. This was used for all TP-M9 cell incubations.

4. DNA Synthesis and Cell Division

Log phase cells were prelabeled for 3-5 generations in M9 medium with 1.0 μCi/ml ^{14}C-thymine (SA 55 mCi/mmole).

Figure 7.--The action spectrum of phototoxicity for the induction of photoproduct(s) of L-tryptophan which are toxic to recA cells (S. typhimurium). The dashed lines show the absorption spectrum of L-tryptophan. The closed circle plot shows the efficiency of monochromatic light for production of toxic photoproducts of L-tryptophan. The bared line is the efficiency of broad spectrum blacklight (310-405 nm) for the production of toxic products from L-tryptophan (212).
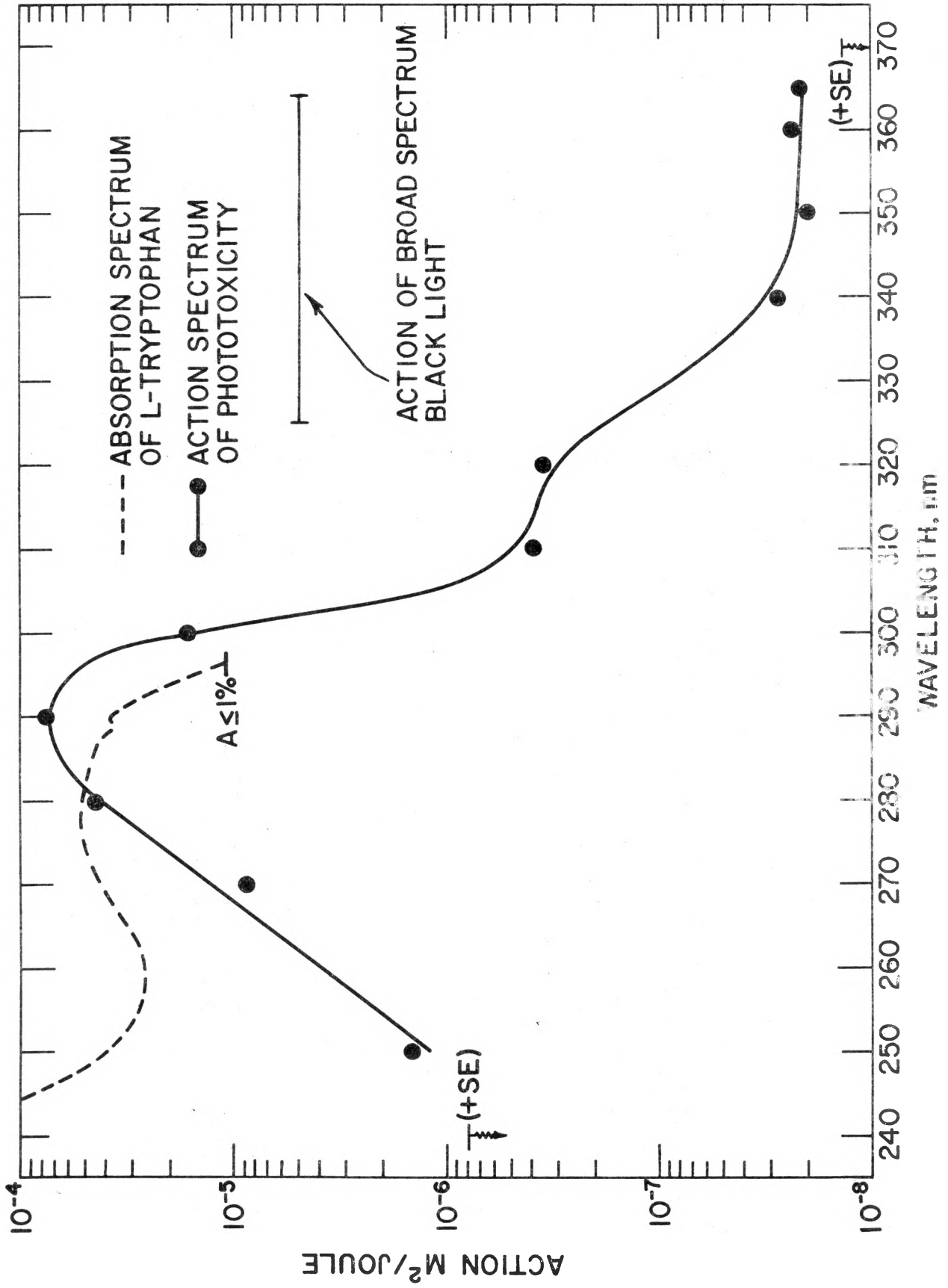
Figure 7

The prelabeled cells were then harvested on membrane
filters and resuspended in M9 with 10 μ Ci/ml ^{3}H-thymidine
(SA 6.7 Ci/mmole). These membrane filters were Millipore
0.22 μ filters which had been washed 3X in 1 liter
volumes of distilled water to remove traces of detergent
from the filters, then autoclaved and stored in sterile
distilled water. This filter washing step was implemented
when we discovered that the filtering step (with unwashed
filters) was introducing a long lag before the cells
continued growth. Washing the filters thoroughly before
use eliminated this growth lag.

To measure DNA synthesis, 20 μl samples were
placed on Whatman 542 filter paper discs saturated with
5% TCA (trichloroacetic acid). The samples were then
washed twice with 5% TCA and once with 95% ethanol in an
ice bath to remove unincorporated TCA soluble counts.
The filter paper discs were dried and placed in scintilla-
tion vials with about 5 ml of 5.5 g/l of Packard Permablend
I in toluene and counted in a Beckman 100 liquid scintilla-
tion counter. Standards were prepared on Whatman 542
filters and counted as above to determine the ^{3}H : ^{14}C
spillover ratios and each sample was normalized.

The response of cell division processes to TP
was determined by removing 0.1 ml of cells at the same
time samples were taken for DNA synthesis determinations,
and placing the cells in 10 ml of 0.1 N HCl for cell

counting and sizing with a modified Coulter counter. This cell counter is a modification of the standard counter with the aperture and electronics selected for accurate counting and sizing of bacteria (214).

5. Analysis of DNA

The single stranded size of prelabeled DNA, and DNA synthesized in the presence of TP, was determined by sedimentation through alkaline sucrose gradients by a modification of the procedure of McGrath and Williams (70). Log phase W3110 cells were prelabeled in M9 medium with 1.0 μCi/ml of ^{14}C-thymine (55 mCi/mmole). The prelabeled cells were harvested at 2 x 10^8 cells/ml on a membrane filter and resuspended in TP-M9 medium with 10 μCi/ml ^{3}H-thymidine (SA 6.7 Ci/mmole). The cells were then allowed to incorporate ^{3}H-thymidine in the presence of TP for 15 minutes at 37°C in the presence of TP. The cells were then harvested on a filter and resuspended in TP-M9, or in M9 medium substituted with 100 μg/ml of cold thymidine. A 0.1 ml sample was removed and placed on ice with KCN-Tris-EDTA stop mix (215) at time zero and various times for five minutes.

To avoid breakage of the DNA, the cells were lysed on top of a 5-20% alkaline sucrose gradient by first layering 0.2 ml of 0.05% sodium dodecyl sulfate in a solution of 0.5 N NaOH to which 50 μl of cells (ca. 1 x 10^7) were added. After allowing 30 minutes for cell lysis

at room temperature, the gradients were centrifuged by using a SW56Ti rotor in a Beckman L-2 65B ultracentrifuge at 35,000 rpm for 95 minutes at 20°C. Eight drop fractions were collected from the bottom of the gradients onto Whatman 17 paper strips by the method of Carrier and Setlow (216). The strips were washed at room temperature once with 5% TCA, and twice with 95% ethanol before being dried and counted as above.

B. THE EFFECT OF TP ON DNA REPAIR

1. Bacterial Strains

The following strains were used in the experiments described below: W3110 wildtype, thy; P3478 polA1 thy; X9247 recA recB thy. All strains used were derived from Escherichia coli K12. The genotypes and phenotypes are listed in Table 1.

2. Growth and Media

Log phase cells were obtained for labeling by 1/20 dilution of cultures which had grown through at least 20 generations of log phase growth as described previously in Section A of Material and Methods. Log phase cells were collected on membrane filters and resuspended 1/20 in fresh M9 medium with 10 μCi/ml of ^{3}H-thymidine (SA 6.7 Ci/mmole). The log phase cells were incubated in labeled medium for 3-5 generations and were harvested for the experiment at 2 x 10^8 cells/ml. When the

cells were to be used for survival curves, they were grown out in medium with no ^{3}H-label and harvested on membrane filters and resuspended in M9 buffer (pH 6.9). The M9 buffered cell suspensions were gently cooled to 4°C by placing the sample tube in a rack on top of ice for a 20 minute period before irradiation. The cells were then irradiated either with a low pressure mercury lamp emitting a large amount of 254 nm, filtered with a 2537A band pass filter, or exposed to X-rays from a 250 KVP - X-ray tube operating at 30 mamp with 0.25 mm Cu and 1.0 mm Al filteration at an approximate dose rate of 2,000 rad/ minute. All of the X-ray dosimetrey, and mechanical operation of the X-ray machine, was conducted by J. Treir, the Argonne Bio-Med X-ray technician (as per ANL regulations). After the cells were irradiated, samples were resuspended in M9 medium or in TP-M9 medium and incubated for 45 minutes at 37°C before being plated on nutrient agar plates in quadruplicate to determine survival by colony forming ability.

Cells which had been labeled in their DNA with ^{3}H-thymidine for use in experiments to test the cells' ability to repair X-ray induced single strand breaks in their DNA were gently resuspended in the following buffer: a) Phosphate Buffered Saline (PBS), pH 7.3 containing the following in g/l: NaH_2PO_4, 0.03197; $Na_2HPO_4.7H_2O$, 0.20655; NaCl, 5. Before exposure the cells were given approximately

15 minutes to cool on ice. The cells were irradiated in small glass dishes in 0.2 ml aliquots and O_2 was blown across the top of the dishes during the X-ray exposure to insure aerobic conditions. The cells were chilled during the X-ray exposure by a small ice bath.

Following the prescribed X-ray exposure cells were diluted into the buffer used during irradiation (PBS), buffered TP-PBS equivalency of 375 µg/ml), M9 medium, or TP-M9 medium (equivalency of 375 µg/ml). An unirradiated control sample was held on ice in buffer, an X-irradiated sample was also held on ice in buffer, the two buffer samples were incubated at room temperature, and the two M9 medium samples were incubated at 37°C. To test for "slow-buffer" repair in polA1 p3478 PBS, and TP-PBS cells were incubated at 37°C. Following the incubation period during which the cells were allowed to attempt repair of the X-ray induced single strand breaks with and without TP present, the samples were placed on ice with KCN-Tris-EDTA stop mix (215) until samples were removed and placed in NaOH-SDS lysis mix on top of 5-20% alkaline sucrose gradients. The cells were allowed 30 minutes lysis time at room temperature before centrifugation.

3. Analysis of DNA Repair

The amount of DNA repair which occurred under each set of conditions for the strains tested was measured by calculating (with a computer program designed for this

purpose) the M_n (number average molecular weight) for each alkaline sucrose gradient profile using T4 DNA as a marker (217). The number of single strand breaks was calculated for each sample by comparison of the Mn_t of the treated cells DNA with the Mn_o of control DNA and the number of SS-breaks/10^8 daltons of DNA determined. The difference between the number of breaks in the incubated samples and the number of breaks in the irradiated control which was held on ice equals the amount of repair which occurred during the incubation. The alkaline sucrose gradients were conducted following the same procedure outlined in Section A.5. of Materials and Methods.

C. INTERACTION OF TP AND MONOCHROMATIC 365 NM RADIATION

1. Bacterial Strains

The following strains were used in the experiments described below: W3110 wildtype, thy, P3478 polA1 thy. All strains used were derived from Escherichia coli K12. The genotypes and phenotypes are listed in Table 1.

2. Growth and Media

Log phase cells were obtained for labeling by 1/20 dilution of cultures which had grown through at least 20 generations of log phase growth as described previously in Section A of Materials and Methods. Log phase cells were collected on membrane filters and resuspended 1/20

in fresh M9 medium with 10 μCi/ml of ^{3}H-thymidine (SA

6.7 Ci/mmole). The log phase cells were allowed to

incorporate label for 3-5 generations before they were

harvested for the experiment at 2 x 10^8 cells/ml. When

the labeling period had ended the cells were again

collected on a filter and washed, then the cells were

resuspended in M9 buffered TP.

Stationary phase cells were obtained by plating

cells on nutrient agar (pre-mixed from Difco) and

incubating the plates for 48 hours at 37°C. The cells

were then scraped from the plate and suspended in M9

buffer or M9-TP buffer at approximately 2 x 10^8 cells/ml.

3. Monochromatic 365 nm Irradiation

The concentration of TP in each experiment was

adjusted by dilution of TP so that 20% of 365 nm light

was transmitted thru a 1.0 cm path as measured on a

Gilford spectrophotometer. This transmittance value was

used to calculate the average incident dose for the cells

irradiated in TP with the following formula:

$\gamma = (Io - I) \ln \frac{Io}{I}$ where γ equals the average incidence

dose rate, Io equals the dose rate at the front of the

sample and I equals the dose rate after traversing 1 cm

of sample.

The cells were grown and labeled in their DNA

as described above. Samples were then suspended in M9

buffer or TP-M9 buffer and irradiated with monochromatic

365 nm light from the previously described xenon mercury

arc lamp and monochromator with Corning glass cutoff

filter #7-51 interposed to reduce scattered light. During

irradiation, the cells were suspended in a jacketed

pyrex cuvette cooled with a constant flow of refrigerated

ethanol to $2^{o}C$, and the cells were mixed by constant

bubbling with air. Following the delivery of various

doses of light, samples were removed and placed on ice

with KCN-Tris-EDTA stop mix (215) to prevent nucleolytic

degradation or repair of the DNA.

4. Analysis of DNA

The size of unirradiated control DNA, and DNA

released from cells which were treated by 365 nm irradia-

tion in M9 buffer, or in TP-M9 buffer, was determined by

sedimentation through alkaline sucrose gradients (70).

Following the irradiation described above 50 µl samples

(ca. 2×10^{6} cells) were removed from the KCN-Tris-EDTA

stop mix and placed on top of a 5-20% alkaline sucrose

gradient in a 0.2 ml layer of 0.05% sodium dodecyl sulfate

and 0.5 N NaOH. After allowing 30 minutes for cell lysis,

the gradients were centrifuged and fractions collected

as described previously in Section A.5. of Materials and

Methods. The Mn (number average molecular weight) was

calculated for each gradient profile. The number of single

strand breaks (alkali-label bonds) induced by the radiation

treatment was calculated by comparison of the Mn values

of the control and irradiated cells. This data was plotted as SS-breaks per 10^8 daltons of DNA verses dose and a slope determined for cells irradiated with and without TP present.

IV. RESULTS

A. THE EFFECT OF TP ON DNA REPLICATION

AND CELL DIVISION

1. Effect of TP on DNA Replication

The rates of ^{3}H-thymidine incorporation into TCA insoluble counts in the presence and absence of TP are shown in Figure 8. TP has a differential effect on DNA replication for W3110 wildtype, AB2500 uvrA, P3478 polA1, and X9247 recA recB. These strains immediately slow or stop DNA synthesis in the presence of TP. However, the effect of TP on DNA synthesis in W3110 wildtype and Ab2500 uvrA is only temporary and the cells recover normal rates of DNA replication within 60 minutes incubation in the presence of TP (Figure 8E).

In contrast, TP immediately terminates DNA replication in P3478 polA1 and X9247 recA recB, and no recovery of DNA synthesis was observed for these strains during the 150 minute incubation period (Figure 8F). Although the pol and rec mutants are deficient in two separate repair functions, both classes of mutation result in X-ray sensitivity, and a reduction in the ability to repair X-ray induced DNA single-strand breaks (218). The incubation of log phase P3478 polA1 cells in TP (at a

Figure 8.--The effect of tryptophan photoproduct
(TP) on DNA synthesis of log phase cells, as determined by
the incorporation of ^{3}H-thymidine into TCA insoluble counts.
The response of the following strains of E. coli is shown:
(A) W3110, thy, wildtype; (B) AB2500 thy, uvrA; (C) P3478
thy, polA1; (D) X9247 thy, recA, recB. The amount of
^{14}C-prelabeled DNA present as TCA insoluble radioactivity
following the TP-M9 medium incubation is shown by the
closed triangle line for each strain tested. The amount
of ^{3}H-thymidine incorporated into TCA insoluble radioacti-
vity during the TP-M9 medium incubation is plotted as closed
circles for each strain tested. Finally, the amount of
^{3}H-thymidine incorporated into TCA insoluble radioactivity
during a parallel incubation in M9 medium with no TP present
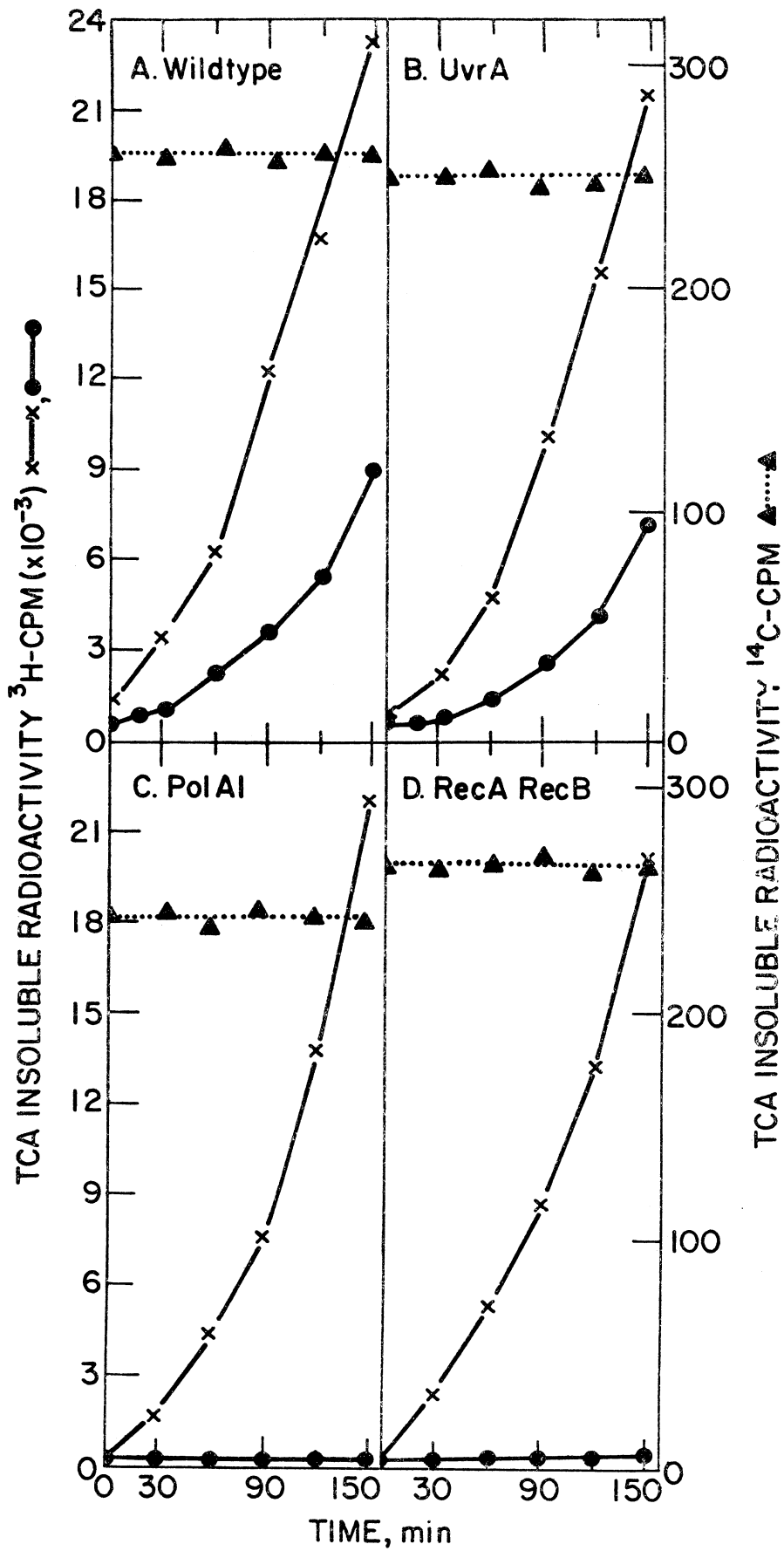is shown by the X----X line for each strain tested.

Figure 8

concentration which kills ca. 90% of a rec population)
results in less than 15% lethality for this strain (Table
1). Thus, the effect of TP incubation on the polA1 strain
is bacteriostatic and removal of the photoproduct allows
this strain to divide normally.

The constancy of the ^{14}C-prelabel throughout the
TP incubation of all strains tested (Figure 8) indicates
that TP does not cause degradation of the parental DNA,
and that concurrent DNA degradation does not account for
the depression of DNA synthesis in the presence of TP.
Moreover, the selective inhibition of DNA synthesis in
mutants which are both sensitive to X-irradiation (recA,
polA1) and reduced in their capability to repair X-ray
induced DNA-single-strand breaks (218), suggests the
possibility that TP might affect DNA replication by slowing
the repair of replication gaps.

2. The Effect of TP on Cell Division

During the DNA synthesis experiments described
above, samples were removed to be counted and sized on
the Coulter counter. The results plotted in Figure 9
show that with the exception of the X9247 recA recB mutant,
cell division and DNA replication remain coupled in the
presence of TP. The response of DNA synthesis (Figure 8)
and cell division (Figure 9) to TP was parallel in all
strains tested except the recA recB double mutant. Whereas
DNA synthesis was inhibited in the polA1 mutant, there was

Figure 9.--The effect of tryptophan photoproduct

(TP) on the increase in relative cell numbers of log phase

cells, as measured by total count with a modified Coulter

counter. The response of the following strains of E. coli

is shown above: (A) W3110 thy, wildtype; (B) AB2500 thy,

uvrA; (C) P3478 thy, polAl; (D) X9247 thy, recA, recB;

(E) W3110 and AB2500 incubated in TP-M9; (F) P3478 and

X9247 incubated in TP-M9. The closed circle plots show

the increase in cell number of W3110 when incubated in TP-

M9 medium. The closed triangle lines show the cell numbers

of AB2500 incubated in TP-M9 medium. The closed squares

are a plot of the increase in cell numbers of P3478 in the

presence of TP. The closed diamonds show the response of

increase in cell numbers for X9247 incubated in TP-M9 medium.

The relative increase in cell numbers of parallel samples

incubated in M9 medium with no TP present is shown by the

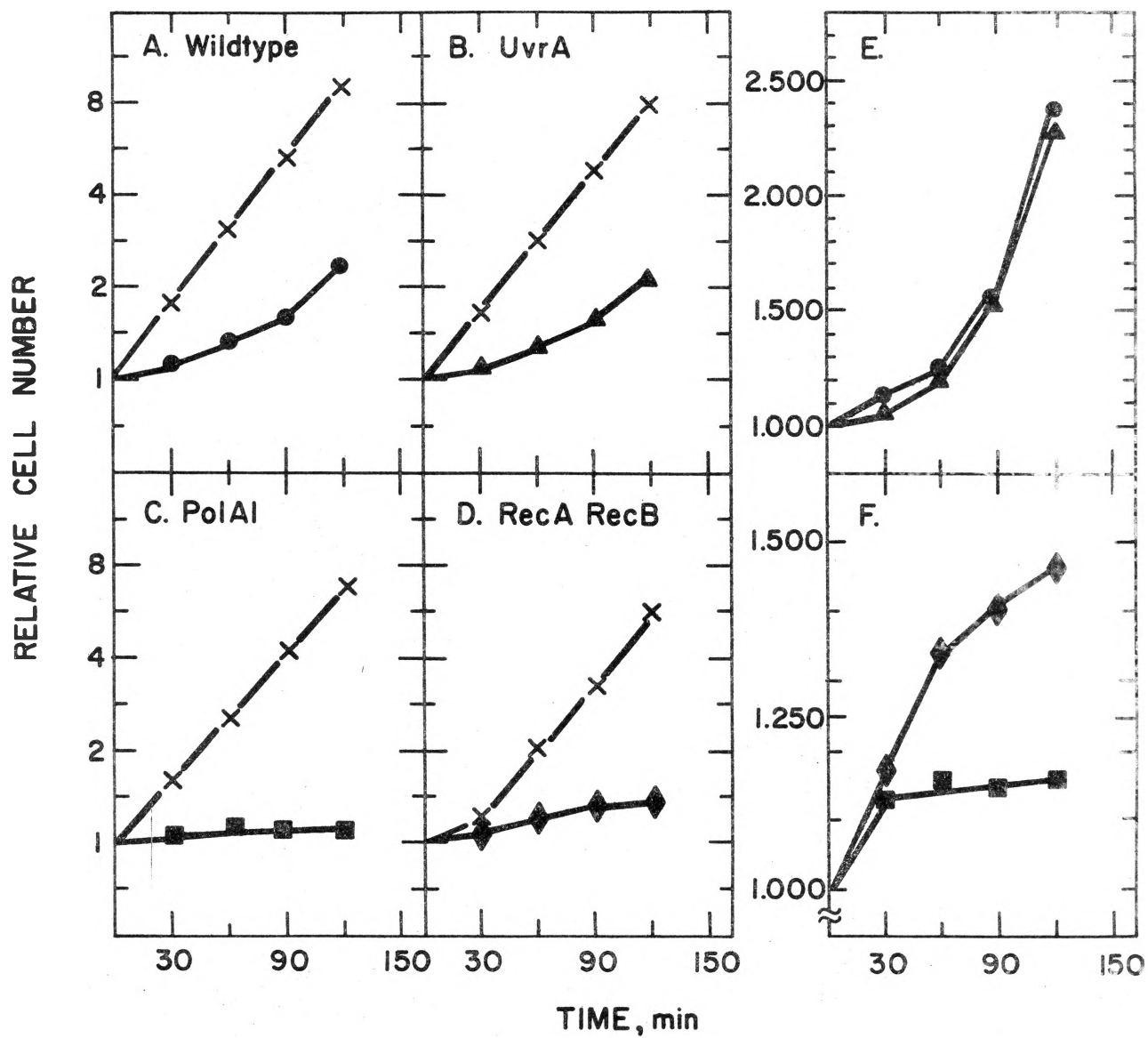X——X line for each strain tested.

Figure 9

no increase in cell numbers after 30 minutes (0.16 total

increase) in the presence of TP. However, during the 150

minute TP incubation of the X9247 recA recB mutant approxi-

mately 0.46 of the cells completed a division with in-

sufficient DNA synthesis to provide complete chromosomes

for these cells. Also, the recA recB mutant continued

to increase in cell numbers throughout the TP incubation

period (Figure 9F) while DNA synthesis was completely

inhibited (Figure 8D). M. Inouye has previously reported

that inhibition of DNA replication in E. coli recA strains

results in the uncoupling of DNA replication from cell

division (219). These results agree with those of M.

Inouye (219) and indicate that TP inhibition of DNA

synthesis in the recA recB mutant results in uncoupling

of DNA replication from cell division.

The cell sizing data in Figure 10 are also con-

sistent with the interpretation that chromosome replication

and cell division are uncoupled only in the rec mutant.

The wildtype and uvrA cells increased in mean cell size

during the first 30 minutes of incubation in TP-M9 medium.

During the TP incubation of wildtype and uvrA, a mean cell

size similar to control cultures incubated in M9 medium

was regained (Figure 10A, B). This recovery of normal

size distribution (Figure 10) is coordinated with the

recovery of DNA synthesis in these strains in the presence

of TP (Figure 8). The polA1 mutant showed no change in

Figure 10.--The effect of tryptophan photoproduct
(TP) on the cell size distribution of log phase cells, as
measured with a modified Coulter counter. The response of
the following strains of E. coli is shown above: (A) W3110
thy, wildtype; (B) AB2500 thy, uvrA; (C) P3478 thy, polA1;
(D) X9247 thy, recA, recB. The size distributions of cells
incubated for zero, 60, 90, and 120 minutes in TP-M9
medium are plotted above for each of the strains tested
as follows: (1) zero time of incubation is plotted as a
solid line, (2) 30 minutes of incubation in TP-M9 is plotted
as a dashed line; 90 minutes is plotted as a dotted line;
and 120 minutes incubation is plotted as a dash-dot-dash
line.

A
Wildtype W3110
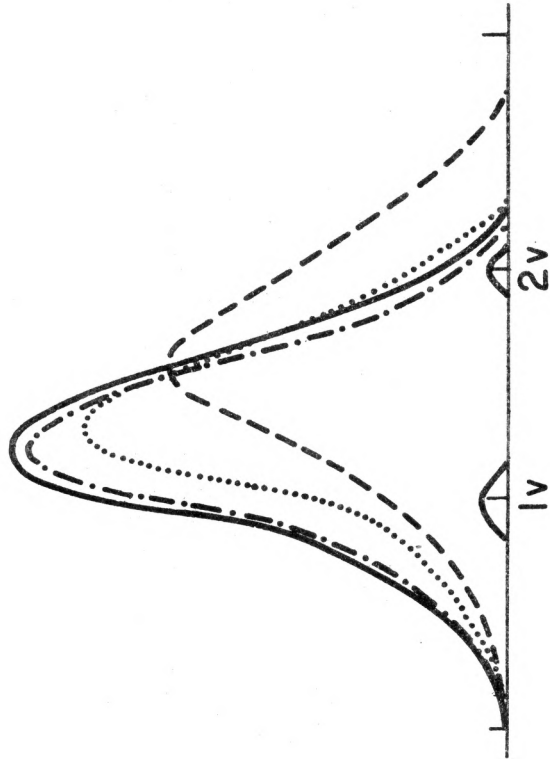
B
uvrA AB2500
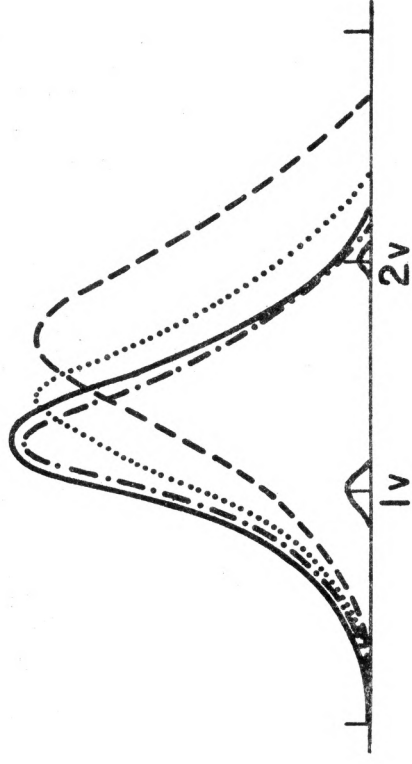
C
polAl P3478
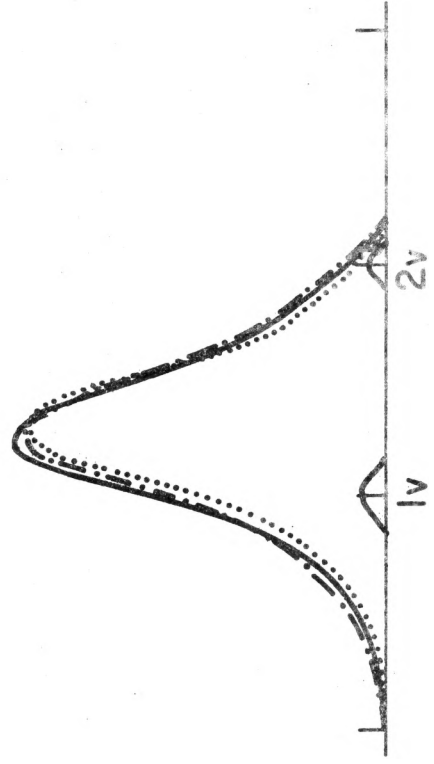
D
RecA RecB X9247
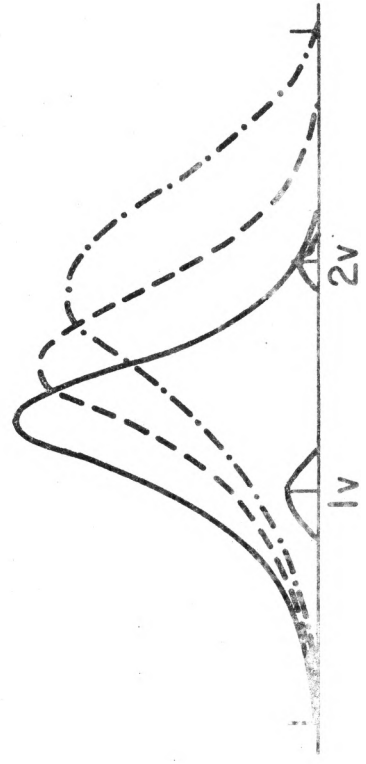
Figure 10

cell size distribution during the TP incubation (Figure 10C), paralleling the complete inhibition of DNA synthesis (Figure 8C) and absence of residual cell division (Figure 9F) observed for the polAl strain. However, the mean cell size of the recA recB mutant increased throughout the TP incubation period (Figure 10D) even though DNA synthesis was completely inhibited in this strain (Figure 8D). Inouye has previously reported that inhibition of DNA replication in a recA strain causes an increase in the mean cell size distribution which contains a sub-population of mini cells (219). Whereas no attempt was made to isolate mini cells from TP incubated cultures of recA recB cells, the observation of an increase in the mean cell size in TP incubated cultures agrees with Inouyes' finding. Thus, the cell sizing data in Figure 10 support the conclusion that TP inhibition of DNA synthesis in the recA recB mutant results in uncoupling of DNA replication from cell division.

3. Effect of TP on Parent and Nascent DNA

Figure 11 shows the sedimentation profiles of ^{14}C-prelabeled DNA, the DNA labeled with ^3H-thymidine in the presence of TP, and the chase profiles of the ^3H-labeled DNA. Intermediate pieces of ^3H-labeled DNA of approximately 7×10^6 daltons [as calculated using T4 DNA as a marker (218)] were observed after labeling in the presence of TP for 15 minutes (Figure 11, triangles).

Figure 11.--Alkaline sucrose gradient sedimentation profiles of DNA from W3110 thy wildtype, labeled or treated as follows: (1) DNA which was prelabeled with ^{14}C-thymine in M9 medium, from cells which were then washed and incubated in TP-M9 medium with ^{3}H-thymidine for 15 minutes, (2) DNA which was synthesized in TP-M9 medium labeled with ^{3}H-thymidine; (3) DNA which had been synthesized in TP-M9 medium and then chased in either M9 medium or TP-M9 medium with cold thymidine. The ^{14}C-prelabeled DNA sedimentation profile is plotted with a dotted line in both Figures 11A and 11B. The sedimentation profile of DNA allowed to incorporate ^{3}H-thymidine in TP-M9 medium for 15 minutes is plotted as a closed triangle line in both Figures 11A and 11B. The sedimentation profile of DNA from cells which had been allowed to incorporate ^{3}H-thymidine in TP-M9 and were then chased in M9 medium for 30 seconds is plotted on the open circle line in Figure 11A. The sedimentation profile of DNA from cells labeled in TP-M9 medium and chased in M9 medium for 60 seconds is plotted on an open square line in Figure 11A. The sedimentation profile of DNA labeled in TP-M9 medium with ^{3}H-thymidine and chased in TP-M9 medium for 2 minutes is plotted on the closed circle line in Figure 11B. The sedimentation profile of DNA labeled in TP-M9 medium, and chased in TP-M9 medium for 5 minutes is plotted on the open diamond line in Figure 11B.

98
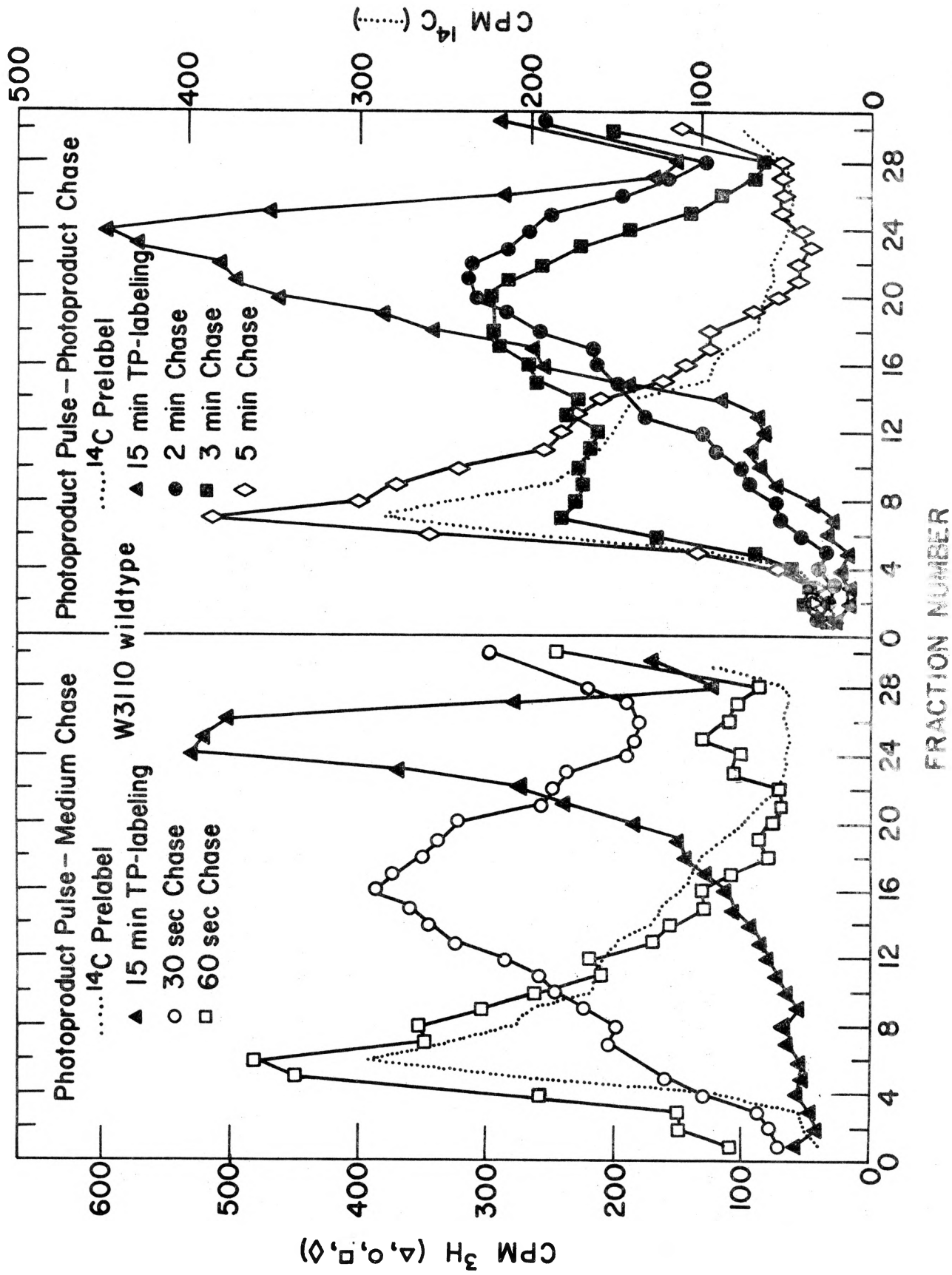
Figure 11

These DNA intermediates do not result from TP activated nucleases since the ^{14}C-prelabeled DNA peaks in the same position as control experiments with no TP present (data not shown). Thus, there is no detectable degradation of parental DNA in wildtype cells during the TP incorporation and chase incubations.

When cells are allowed to incorporate ^{3}H-thymidine for 15 minutes in the presence of TP, small pieces of DNA are made. These DNA-pieces correspond in size to late replicative intermediates reported by Okazaki and others (79, 220). The appearance of these small pieces of DNA in cells incubated in TP could be explained by one of the following hypotheses: 1) TP could cause the accumulation of replicative intermediates which would normally be rapidly joined into high molecular weight DNA by inhibiting a step(s) required for the closure of these gaps; 2) TP might interfere directly with the replication fork by intercalating between strands in front of the replication fork thus producing a gap in the nascent DNA; 3) TP might act by stimulating a nuclease which degrades the DNA into pieces of approximately 7×10^6 daltons. To distinguish among these possibilities, chase times of nascent DNA pieces synthesized in the presence of TP into high molecular weight DNA were compared for samples chased in the presence and absence of TP. The nascent DNA pieces which were chased in TP-M9 medium required 5 minutes of

incubation before the DNA pieces were returned to the same position on the alkaline sucrose gradient as the ^{14}C-prelabeled parental DNA (Figure 11, diamonds). In contrast, samples chased without TP present required only 60 seconds of chase time to regain the control position on the alkaline sucrose gradient (Figure 11, squares). A comparison of these chase times indicates that TP causes an 80% reduction in the rate of gap closing events. This result is consistent with the first hypothesis that the appearance of low molecular weight DNA in cells labeled in the presence of TP represents the accumulation of replicative intermediates as a result of the inhibition of some step(s) in the closure of replication gaps.

B.   THE EFFECT OF TP ON DNA REPAIR

1.   Effect of Postirradiation Incubation in TP-M9 on UV
     and X-ray Survival

Log phase cells were preirradiated with 20 erg/mm^2 of far-UV (254 nm) in M9 buffer. These preirradiated cells were then placed in TP-M9 medium (various concentrations) and incubated at 37°C for 45 minutes before plating in quadruplicate on nutrient agar plates to determine the colony forming ability of each strain tested. The surviving fraction of the following strains is plotted in Figure 12: a) AB1157 wildtype; b) AB1886 uvrA; c) AB1884 uvrC. The dark incubation of these strains in

Figure 12.--The effect of a nonlethal dose of far-UV radiation (20 erg/mm^2) on the survival of the following strains in tryptophan photoproduct (TP); AB1886 uvrA; AB1157 wildtype; AB1884 uvrC. The surviving fraction following the irradiation of washed log phase cells and postirradiation incubation in medium substituted with various concentrations of TP is plotted as closed squares for AB1886 uvrA; closed circles for AB1157 wildtype; and closed triangles for AB1884 uvrC.
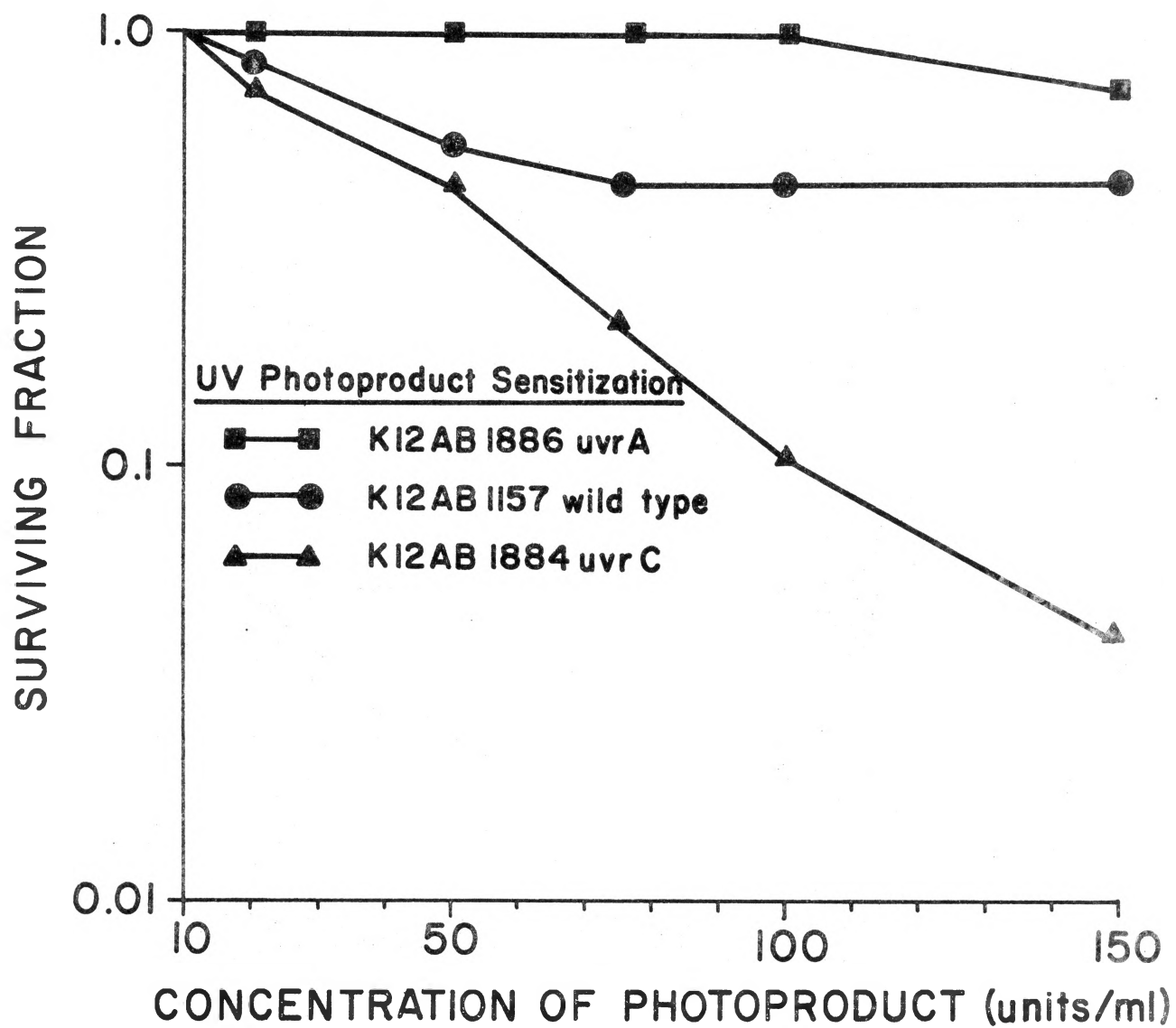
Figure 12

TP-M9 medium with no preirradiation does not significantly
reduce the viability of the population (less than 10%
lethality) (213). However, postirradiation incubation in
TP-M9 medium differentially sensitized these strains to
a non-lethal dose of far-UV. The results in Figure 12
show that AB1886 uvrA was not sensitized to UV irradiation
by the TP-M9 incubation (Figure 12, squares). However,
wildtype AB1157 was killed to approximately 57% survival
by incubation in 100 units/ml of TP following far-UV
irradiation (Figure 12, circles), and 1884 uvrC was
sensitized to more than a log reduction in survival by
incubation in 100 units/ml of TP following far-UV irradia-
tion (Figure 12, triangles). Since AB1886 uvrA is
deficient in a dimer-specific endonuclease which is
required for the efficient induction of incision "nicks",
and this strain is less sensitive to the UV-TP treatment
than wildtype cells, the TP-sensitization of cells to
far-UV may be dependent on the induction of breaks in the
DNA during excision repair. The sensitivity of strain
AB1884 uvrC to the UV-TP treatment supports this hypothe-
sis. The uvrC strain can make dimer-specific endonucleoly-
tic "nicks" but cannot excise the dimer. Therefore, AB1884
can make incision nicks during attempts to repair the
dimers and the presence of these nicks may be responsible
for the UV-TP sensitization of this strain.

Figure 13 shows the effect of incubating W3110 wildtype cells in TP-M9 medium (150 units/ml) for 60 minutes at 37°C after the cells were exposed to X-irradiation. Log phase W3110 cells were washed and irradiated in M9 buffer at 4°C aerobically, diluted into TP-M9 medium and incubated for 60 minutes. Following the postirradiation incubation, the cells were plated on nutrient agar in quadruplicate and incubated at 37°C for 48 hours to determine survival by the ability to form colonies. The survival of W3110 held in buffer following irradiation is shown in the closed circle plot in Figure 13. The postirradiation incubation of W3110 wildtype in TP-M9 medium enhanced the lethal effect of X-irradiation by approximately 1.9 fold on the dose axis as shown in Figure 13 (closed triangles). This X-ray sensitization of wildtype cells suggests that TP may act against some step(s) required to repair DNA-single strand breaks. Therefore, the sensitization observed for both far-UV and X-ray lethality by postirradiation incubation in TP suggests that TP acts as a DNA repair inhibitor.

2. Effect of TP on the Repair of X-ray Induced Single Strand Breaks in DNA

Figure 14 shows the sedimentation profiles of DNA released from W3110 wildtype cells which have been exposed to 20 krad of X-irradiation administered at 0°C and treated by holding at 0°C; postirradiation incubation in

Figure 13.--The effect of postirradiation incuba-
tion of AB1157 wildtype cells in tryptophan photoproduct
substituted medium (TP-M9) on X-ray survival. The closed
triangle plot represents the survival of AB1157 cells in-
cubated in TP-M9 medium at 37°C for 60 minutes prior to
plating (in quadruplicate) on nutrient agar to determine
survivors by colony forming ability. The closed circle
plot indicates the survival of AB1157 exposed to X-irradia-
tion and held in buffer on ice before plating for survival.
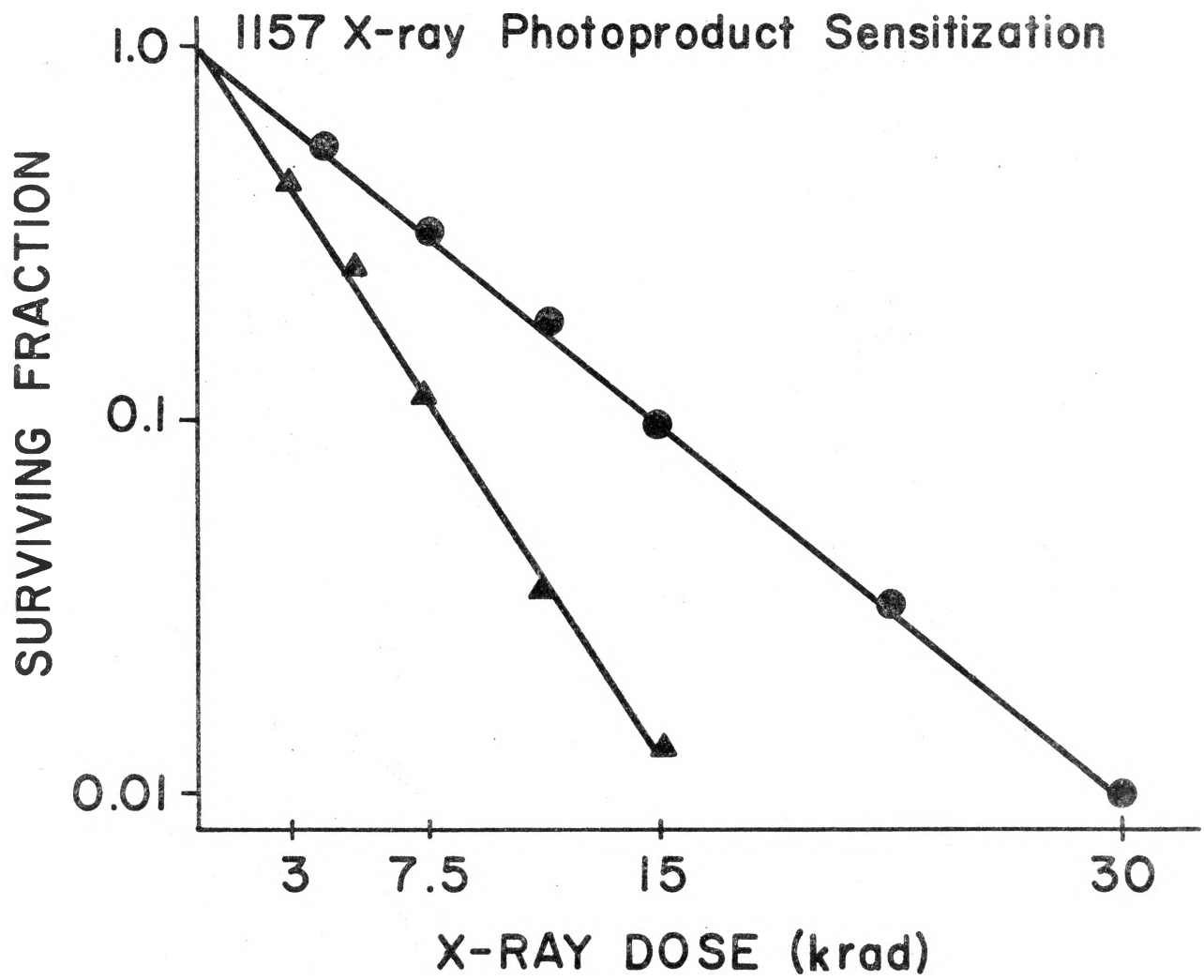
Figure 13

Figure 14.--The effect of TP on the repair of X-ray induced single strand breaks in DNA of wildtype W3110. The alkaline sucrose gradient profile of DNA released from W3110 cells is shown for DNA which was centrifuged at 35,000 rpm in a SW56Ti rotor for 95 minutes at 20°C. The open circle profile is the sedimentation profile of untreated DNA released from labeled W3110 cells which had been held in buffer (PBS) on ice. The open square profile is the sedimentation profile of DNA released following 20 krads of X-irradiation administered at 0°C and held at 0°C in PBS (aerobic). The closed circle profile shows the sedimentation of DNA released from W3110 cells exposed to 20 krads of X-irradiation and then incubated in M9 medium at 37°C for 30 minutes. The closed squares are a plot of the profile of DNA released from W3110 cells exposed to 20 krads of X-irradiation then incubated in TP-M9 medium at 37°C for 30 minutes. The open triangle shows the sedimentation profile of DNA released from W3110 cells exposed to 20 krads of X-irradiation followed by incubation in PBS at 25°C for 20 minutes. The closed triangle profile represents the sedimentation profile of DNA released from W3110 cells exposed to 20 krads of X-irradiation then incubated in TP-PBS at 25°C for 20 minutes.
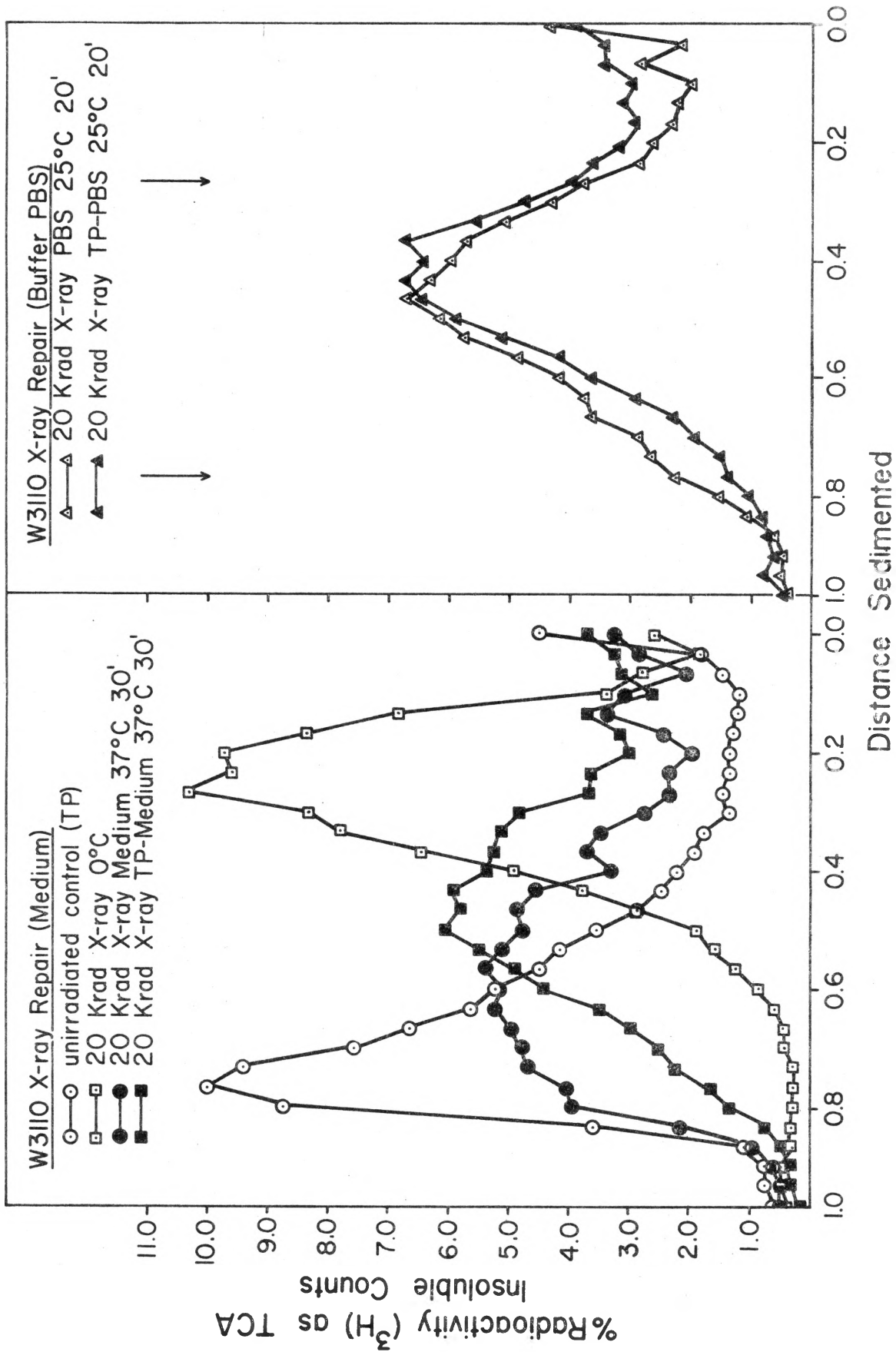
Figure 14

PBS pH 7.3 for 20 minutes; postirradiation incubation in
TP-PBS pH 7.3 for 20 minutes; postirradiation incubation
in M9 medium at 37°C for 30 minutes; postirradiation
incubation in TP-M9 medium at 37°C for 30 minutes; and
untreated control cells held on ice. The single strand
breaks induced in the DNA of W3110 cells by 20 krads of
X-irradiation at 0°C is shown by the open square plot
in Figure 14. The Mn (Number Average Molecular Weight)
was calculated using T4 DNA as a marker (130, 218). The
number average molecular weight was calculated by the
following formula: $Mn = \Sigma Ri/\Sigma(Ri/Mi)$ where Ri is the
percentage total TCA insoluble radioactivity in fraction
i. The number average molecular weight (Mn) was used to
calculate the number of ss-breaks per 10^8 daltons of
DNA by the following formula: $\beta = 10^8(\frac{1}{Mn_t} - \frac{1}{Mn_o})$ where
Mn_t equals the determined Mn for the treated sample, and
Mn_o equals the Mn determined for the untreated control
DNA, and β equals the number of single strand breaks per
10^8 daltons of DNA (221). The Mn calculated for each of
the profiles in Figure 14 equal the following (x 10^6
daltons): (1) unirradiated control (open circles) 115.3;
(2) 20 krad X-irradiation (open squares), 6.8; (3) 20 krad
X-irradiation and incubation in PBS at 25°C (open triangles),
30.5; (4) 20 krad X-irradiation and incubation in TP-PBS
at 25°C (closed triangles), 25.0; (5) 20 krad X-irradiation
and incubation in M9 medium at 37°C (closed circles), 90.4;

(Type II) of X-ray induced ss-breaks in DNA (222). Since 173.00 ss-breaks/ss-genome are available for repair (Mn 6.8), substraction of the number of breaks left in each repair profile indicates that the following number of breaks were repaired (ss-breaks/ss-genome): 143 in PBS; 134 in TP-PBS; 170 in M9 medium; and 150 in TP-M9 medium. Since $polA1^+$-mediated buffer repair occurs during the medium incubation, the number of breaks repaired in PBS are subtracted from the number repaired in medium to yield the number of breaks which require Type III (Medium Dependent) repair (85). This calculation indicates that 27 ss-breaks/ss-genome require medium conditions to complete repair, and that only 7 ss-breaks/ss-genome are repaired in TP-M9 medium which are not repaired in PBS (Type II). Therefore, TP (at this concentration) inhibits medium-dependent (Type III) repair by 75%. The effect of TP on medium-independent (Type II) repair in PBS can be calculated directly from the number of breaks repaired with TP present and in PBS with no TP present. This calculation indicates that TP inhibits Type II polA1 mediated repair by only 6%. There is some overlap of these repair sectors, in that polA1 strains can slowly repair breaks in PBS when incubated at 37°C (83). Preliminary results of Youngs and Smith (83) indicate that DNA polymerase III activity is required for "slow" buffer repair in polA1 strains. Since there is an overlap of

Type II and Type III repair, the 6% inhibition of Type II
repair in wildtype cells (TP-PBS incubated) may be the
result of the action of TP on the activity of some repair
process not involving DNA polymerase I activity.

2.1 The Effect of TP on Repair of X-ray-induced
Single Strand DNA Breaks in a polA1 Strain.

The results in Figure 15 show the effect of TP
on "slow" buffer repair in P3478 polA1 incubated in PBS
at 37°C. When P3478 polA1 is incubated in PBS for
extended periods at 37°C, many of the breaks that are
normally repaired very rapidly at 25°C in polA1$^+$ may now
be "slowly" repaired by other DNA polymerases in the cell
(83). The sedimentation profiles in Figure 15 were used
to calculate Mn values as described above in Results
Section B.2. The calculated Mn values for the profiles
in Figure 15 equal the following (x 10^6 daltons): (a)
unirradiated control (open circles) 129.1; (b) 16.9 krad
X-irradiation (open squares) 6.7; (c) 16.9 krad X-
irradiation and incubation in PBS 37°C 20 minutes (open
triangles) 30.5; (d) 16.9 krad and incubation in TP-PBS
37°C 20 minutes (closed triangles) 24.9; (e) 16.9 krad
and incubation in PBS 37°C 45 minutes (open diamonds)
36.7; (f) 16.9 krad X-irradiation in TP-PBS 37°C 45 minutes
(closed diamonds) 24.9. Calculation of the number of
ss-breaks/ss-genome as described previously indicates that
the following number of breaks were repaired (ss-breaks/

Figure 15.--The effect of TP on "slow-buffer" re-
pair of DNA following exposure of P3478 polA1 to 16.9 krads
of X-irradiation. The open circle profiles show the
sedimentation profile of DNA released from P3478 cells
labeled in their DNA ^{3}H-thymidine and held on ice. The
open square profile indicates the sedimentation profile
of DNA released from labeled P3478 cells following 16.9
krads of X-irradiation administered at 0^OC in PBS (aerobic),
then held on ice until being placed on top of the gradient.
The open triangle plot is the sedimentation profile of DNA
released from P3478 cells exposed to 16.9 krads of X-
irradiation then incubated at 37^OC for 20 minutes in PBS.
The closed triangle profile shows the sedimentation of DNA
released from P3478 cells following 16.9 krads of X-irra-
diation and incubation in TP-PBS for 20 minutes at 37^OC.
The open diamonds show the sedimentation profile of simi-
larly irradiated P3478 cells following 45 minutes incuba-
tion in PBS at 37^OC for 45 minutes. The closed diamond
profile shows the sedimentation of DNA released from
similarly irradiated P3478 cells following 45 minutes of
incubation in TP-PBS at 37^OC.

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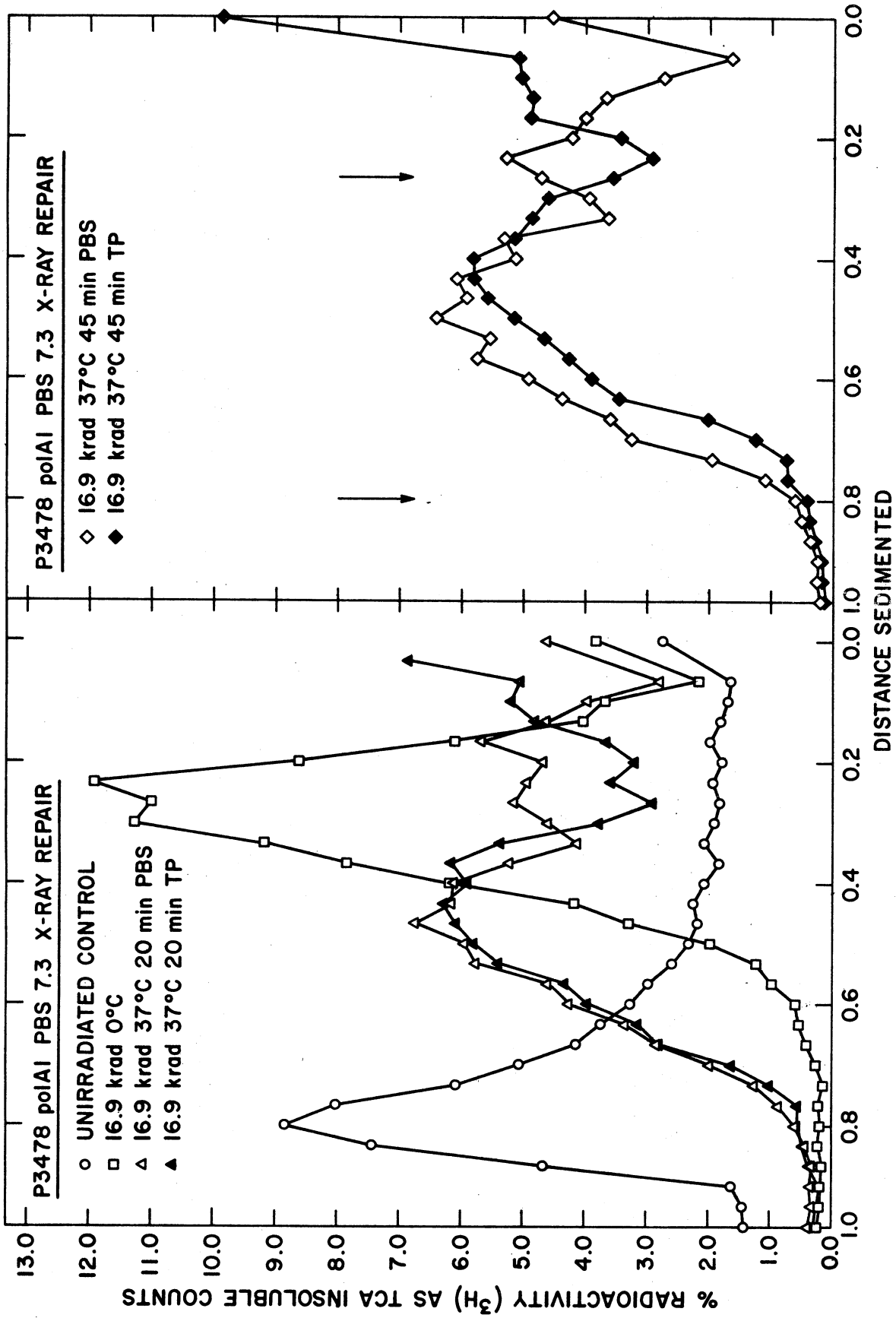
Figure 15

ss-genome): (a) PBS 20 minutes 144; (b) TP-PBS 20 minutes
135; (c) PBS 45 minutes 151; (d) TP-PBS 45 minutes 135
(175 ss-breaks/ss-genome were available for repair).
Following 20 minutes of incubation of P3478 polA1, TP had
inhibited "slow" buffer repair in this strain by 6%,
and following 45 minutes incubation, TP had inhibited this
"slow" buffer repair made by 10%. Since the 45 minute
incubation of this strain in PBS at 37°C allows "slow"
buffer repair to occur to a maximum for these conditions,
these results indicate that TP is not a strong inhibitor
of the processes required for "slow" buffer repair in
polA1 strains. If Youngs and Smith's (83) preliminary
findings are correct (i.e., that DNA polymerase III
activity is required for buffer repair in a polA1 strain),
then these data suggest that TP does not act by inhibiting
the polymerization activity of DNA polymerase III.

The results in Figure 16 show the effect of TP
on medium dependent repair in P3478 polA1. When P3478
polA1 is incubated in M9 medium following a dose of X-
irradiation at 37°C, DNA-single strand breaks are closed
by Type III repair processes (83). However, during this
repair a competing DNA degradation takes place producing
high counts at the top of the gradient (83). This DNA
degradation complicates the interpretation of results.
However, qualitative results on Type III repair in this
strain are useful since no "background" polA1^{+} repair can

Figure 16.--The effect of TP on the medium-dependent repair of X-ray induced single strand breaks in DNA. P3478 polA1 cells were labeled in their DNA and exposed to 16.9 krads of X-irradiation in PBS at 0°C (aerobic), then incubated in M9 medium at 37°C with or without TP present. The open circle profile indicates the sedimentation of DNA released from P3478 cells held on ice (untreated) until being placed on top of an alkaline sucrose gradient for lysis. The open square plot shows the profile of DNA released from P3478 cells exposed to 16.9 krads of X-irradiation at 0°C then held on ice until they were placed on top of an alkaline sucrose gradient. The closed circle plot indicates the profile of DNA released from P3478 cells exposed to 16.9 krads of X-irradiation followed by 30 minutes of incubation in M9 medium at 37°C. The closed square profile indicates the sedimentation of DNA released from P3478 cells exposed to 16.9 krads of X-irradiation then incubated in TP-M9 medium at 37°C for 30 minutes.
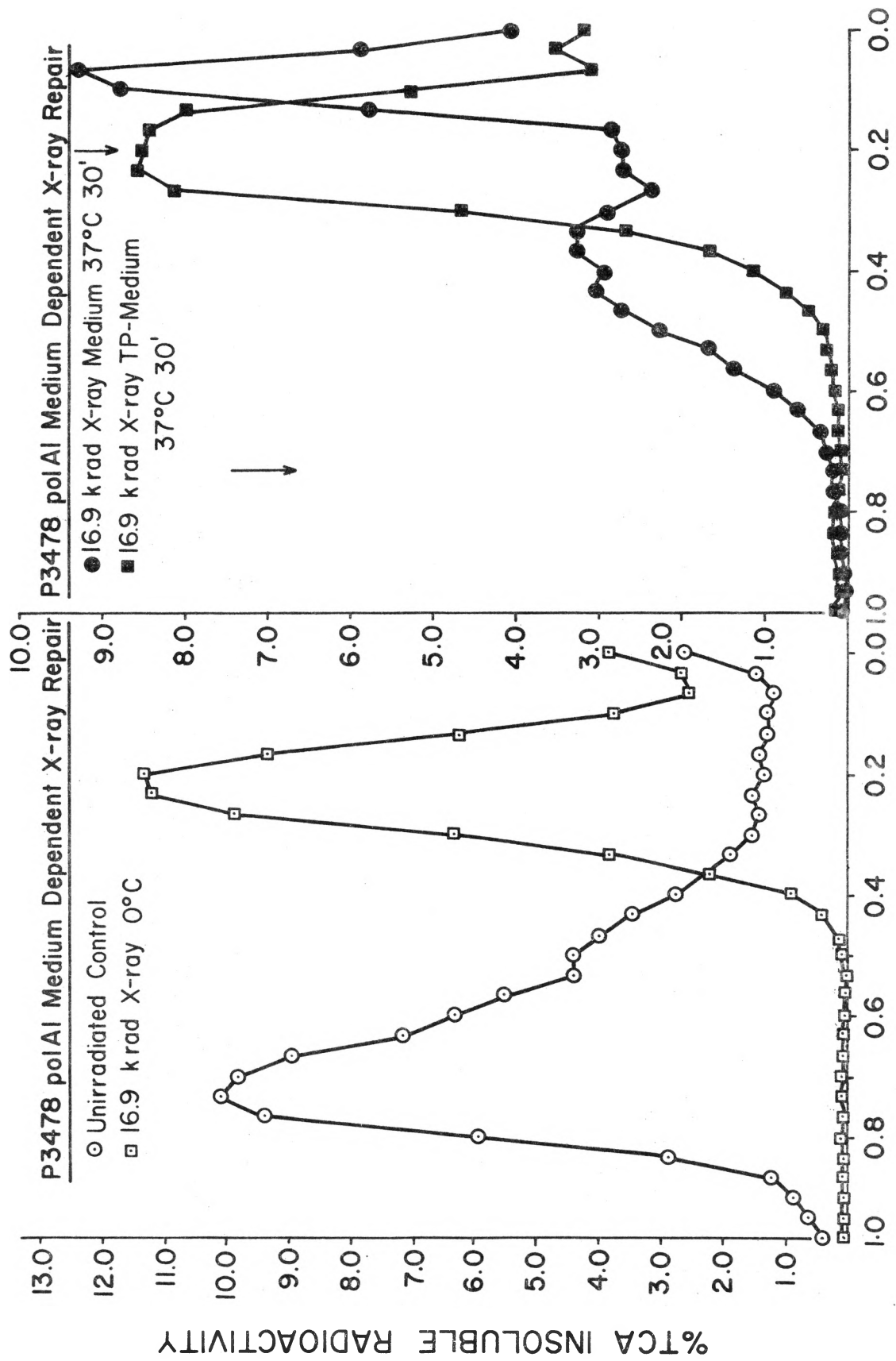
Figure 16

occur. The Mn values calculated from the profiles in Figure 16 are equal to the following (x 10^6 daltons): (a) unirradiated control (open circles) 102; (b) 16.9 krad X-irradiation (open squares) 6; (c) 16.9 krad X-irradiation and 30 minutes at 37°C in M9 medium (closed circles) 23; (d) 16.9 krad X-irradiation and 30 minutes at 37°C in TP-M9 medium (closed squares) 7. Calculating the number of ss-breaks/ss-genome indicates that the following number of breaks were repaired (ss-breaks/ss-genome): (a) M9 medium 37°C 30 minutes, 137; (b) TP-M9 medium 37°C 30 minutes, 8; 180 ss-breaks/ss-genome were available for repair. This indicates that TP inhibits Type III repair in P3478 polAl by 94%. It is also important to note that while the polAl cells incubated in M9 medium showed the characteristic DNA degradation products as oligonucleotides at the top of the gradient (closed circles), the samples incubated in TP-M9 medium did not carry out Type III repair and no degradation products appeared at the top of this gradient (closed squares). This parallel inhibition of Type III repair in the polAl mutant and an inhibition of DNA degradation suggests that the TP inhibition of Type III repair might involve the inhibition of a nuclease required for medium-dependent repair.

C. INTERACTION OF TP AND MONOCHROMATIC 365 NM RADIATION

1. TP-365 nm Synergism for the Induction of "Alkali-
    Labile Bonds" in Bacterial DNA

The results in Figure 17 show the rate of induction
of single strand breaks in bacterial DNA of cells exposed
to 365 nm radiation with and without TP present in M9, or
Tris-EDTA buffer. The alkaline sucrose gradient profiles
were plotted and the Mn calculated as previously described.
The number of ss-breaks/10^8 daltons was calculated from
these Mn values and plotted as number of ss-breaks/10^8
daltons DNA vs. dose of 365 nm irradiation. The closed
triangle plot in Figure 17A shows the rate of induction
of single strand breaks in the DNA of W3110 cells suspended
in a solution of TP-M9 (concentration adjusted to 20%T
at 365 nm), and irradiated with monochromatic 365 nm
light at 2°C. The open triangle plots indicate the number
of ss-breaks/10^8 daltons when W3110 cells are irradiated
in a TP-Tris-EDTA solution (same concentration) with
monochromatic 365 nm light at 2°C. The 0.005 M Tris-
0.001 M EDTA (ethylenediaminetetraacetic acid) buffer
pH 8.0 was used to inhibit any concomitant DNA repair
which migh occur during the irradiation. However, the
results in Figure 17A indicate that the presence of this
inhibitor did not influence the rate of break induction
in cells irradiated with TP present (open triangles). The
slope calculated for the rate of the linear induction of

Figure 17.--TP-365 nm synergism for the induction
of "alkali-labile bonds" in bacterial DNA. The closed
triangle plot in Figure 17A shows the rate of induction
of "alkali-labile bonds" in bacterial DNA of W3110 cells
irradiated in TP-M9 buffer (conc. 20%T at 365 nm) with
monochromatic 365 nm radiation plotted as single strand
breaks/10^8 daltons. The open triangle plot in Figure 17A
indicates the rate of 365 nm induced "alkali-labile bonds"
in DNA released from W3110 cells irradiated in TP-Tris-
EDTA (conc. 20%T at 365 nm). The dashed line shows the
rate of induction of "alkali-labile bonds" in DNA released
from W3110 cells following 365 nm irradiation in M9 buffer.
The closed squares in Figure 17B show the rate of induction
of "alkali-labile bonds" in DNA released from W3110 cells
irradiated in Tris-EDTA buffer. The dashed line in Figure
17B shows the rate of induction of "alkali-labile bonds"
in bacterial DNA in buffered TP (conc. 20%T at 365 nm).

A. TP + 365NM Irradiation

$k_{TP} = 1.59 \times 10^{-5}$ ss-breaks/
10^8 dalton/JM^{-2}

$$\frac{k_{TP}}{k_{buffer}} = 11.53$$

B. 365NM Irradiation

$k_{buffer} = 1.38 \times 10^{-6}$ ss-breaks/
10^8 dalton/JM^{-2}

J/M^2 (x10^{-5})

NUMBER OF SS-BREAKS/10^{8}DALTONS DNA

Figure 17

breaks in the DNA of cells irradiated with 365 nm light in TP-M9 or TP-Tris-EDTA buffer equals the following: k_{TP} = 1.59 x 10^{-5} ss-breaks/10^8 daltons/JM^2. It should be noted that while this difference in sedimentation rate on the alkaline sucrose gradient is described as single strand breaks, in this case the breaks might be some chemical change in the DNA which causes the DNA molecule to be sensitive to the alkaline conditions in the gradient (alkali-labile bonds).

The results in Figure 17B show the rate of induction of ss-breaks/10^8 daltons/JM^2 of W3100 cells labeled in their DNA and irradiated with monochromatic 365 nm light in M9 buffer or Tris-EDTA buffer with no TP present. The closed square plot indicates the number of single strand breaks induced in the DNA of W3110 cells irradiated in M9 buffer, and the open squares indicate the number of DNA-single strand breaks induced by irradiation of cells in Tris-EDTA. It is clearly indicated that concomitant DNA repair does not alter the rate of ss-break induction since the Tris-EDTA buffer did not significantly change the number of breaks induced by 365 nm irradiation. The slope calculated for the linear induction of ss-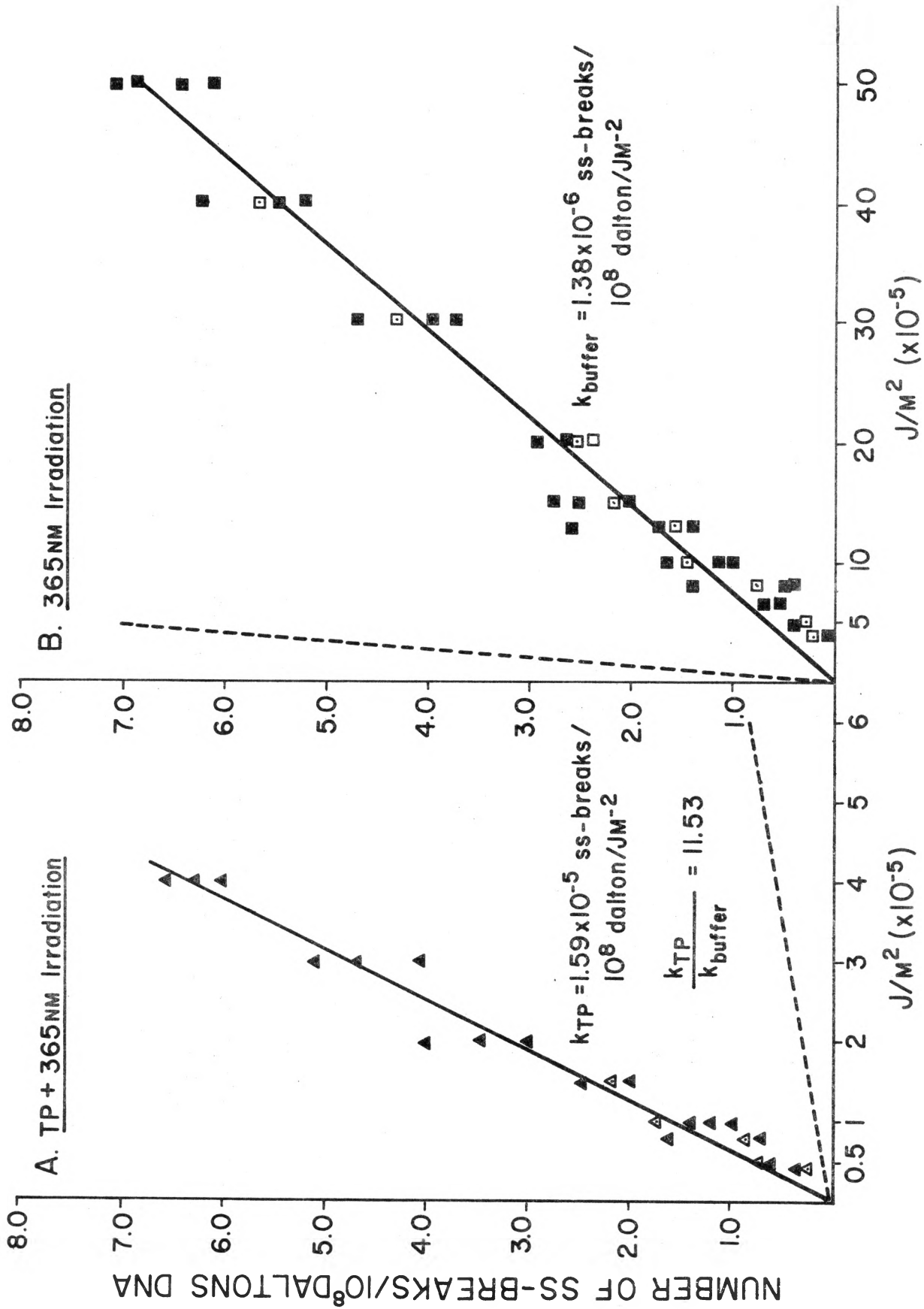breaks in the DNA of cells irradiated with 365 nm light in M9 or Tris-EDTA buffer equals the following: k_{buffer} = 1.38 x 10^{-6} ss-breaks/10^8 daltons/JM^2. It should be noted that these ss-breaks might be alkali-labile bonds. The

"alkali-labile bond" induction ratio for TP-M9 is $k_{TP}/$
k_{buffer} = 11.53. This measured rate of break induction
by 365 nm irradiation of cells in M9 buffer is in close
agreement with the value reported by Tyrrell et al. (211).

2. TP-365 nm Synergism for the Inactivation of P3478 polAl (Stationary Phase) Cells

The results in Figure 18 show the effect of TP
on the lethality of 365 nm radiation for stationary phase
P3478 polAl cells. The stationary phase cells were taken
from the surface of a nutrient agar plate and suspended
in TP-M9 buffer (concentration adjusted to 30%T at 365 nm)
or in M9 buffer. The open square plot in Figure 18 shows
the surviving fraction of stationary phase P3478 cells
suspended in M9 buffer and treated with 365 nm radiation.
The open circle plot shows the surviving fraction P3478
exposed to monochromatic 365 nm light while suspended in
TP-M9 buffer. The results in Figure 18 indicate that
TP-365 nm induced lesions in the DNA ("alkali-labile bonds")
enhance the lethal effect of 365 nm inactivation of P3478
by approximately 2.5 fold on the dose axis. This result
indicates that the TP-365 nm induced lesions in the DNA
also play a role in the inactivation of P3478 polAl,
suggesting that these lesions in the DNA can be lethal to
the cell.

Figure 18.--The synergistic inactivation of P3478 polA1 by TP-365 nm radiation. The open square plot shows the inactivation of P3478 polA1 stationary phase cells treated with 365 nm irradiation in M9 buffer and plated in quadruplicate on nutrient agar to determine survival by colony forming ability. The open circle plot indicates the survival of stationary phase P3478 polA1 cells irradiated with 365 nm light in TP-M9 buffer (conc. 20%T at 365 nm) and plated in quadruplicate to determine survival by colony forming ability.
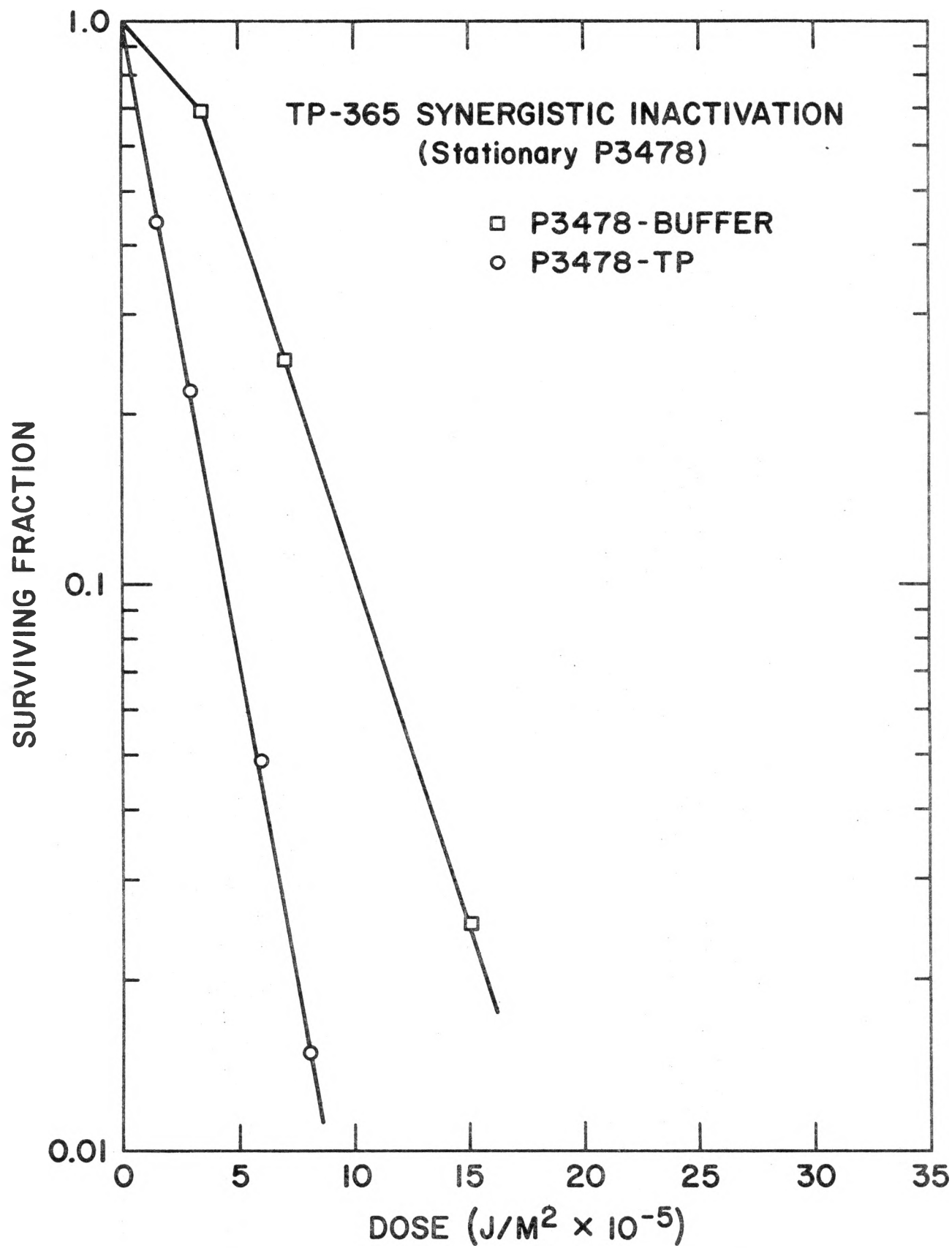
Figure 18

V. DISCUSSION

A. THE EFFECT OF TP ON DNA REPLICATION
AND CELL DIVISION

It is clear from the following observations that
TP has a differential effect on DNA replication in mutants
sensitive to single strand gaps in DNA: (a) DNA replica-
tion in wildtype cells first slowed and then resumed a
normal rate during the TP incubation; (b) uvrA cells
slowed DNA replication in TP and the cells recovered
similar to WT; (c) polA1 cells stopped DNA replication
immediately in the presence of TP; (d) recA recB double
mutant cells stopped DNA replication immediately in the
presence of TP. Therefore, DNA replication is especially
sensitive to TP in mutants (polA1, recA recB) deficient
in the repair of single strand breaks in their DNA. The
special sensitivity of DNA replication to TP in these
mutants suggests that TP inhibits a repair function
required for rapid gap closure, other than DNA polymerase
I activity. There are several steps at which inhibition
of a gap repair function might selectively stop DNA
synthesis in mutants sensitive to single strand breaks.
These steps include: (a) the inhibition of a DNA polymerase
other than polA1 gene product (i.e., polII or polIII); (b)

the inhibition of nucleolytic removal of RNA primers;
(c) the inhibition of ligase joining of completed replication intermediates. The inhibition of replication gap closure would be expected to be more effective in mutants defective in recA or polA1 activity than in cells with normal gap repair abilities.

The inhibition of DNA replication in E. coli B/r normally allows, at most, only a 25% residual increase in cell numbers (223, 224). In polA1 cells, cell division terminates immediately when DNA is inhibited (225, 226). However, Inouye has reported that inhibition of DNA replication (by nalidixic acid, or thymine starvation) in an E. coli recA strain results in more than 25% residual division, an increase in mean cell size, and uncoupling of DNA replication from cell division (219). Inouye attributes this uncoupling to a pleiotrophic effect of the recA mutation on cell division (219). The effect of TP inhibition of DNA synthesis on cell division in the recA recB and polA1 mutants is in agreement with this finding. The conclusion that TP inhibition of DNA synthesis in the recA recB mutant results in uncoupling is supported by the following facts: (a) TP inhibition of DNA replication in the polA1 mutant caused cell division to terminate within 30 minutes, but did not cause an increase in mean cell size, (b) in contrast, TP inhibition of DNA replication in the recA recB mutant did not

terminate cell division and there was a steady increase in cell size throughout the TP incubation, (c) the viability of the rec mutant was substantially reduced during the TP treatment while the pol strain remained viable during the TP incubation.

The analysis of nascent DNA synthesized in TP-M9 medium, using alkaline sucrose gradients, provides evidence that TP inhibits some step(s) necessary for closing replication gaps. Since even late replication intermediates are difficult to detect in wildtype cells growing in glucose at 37^OC (220), the appearance of nascent pieces of DNA during the first 15 minute TP labeling period suggests that TP might inhibit replication gap closure. This low molecular weight nascent DNA could also result from direct interference with the replication fork if TP acts on the DNA in front of the replication fork (hypothesis 2), or by TP stimulation of nuclease (hypothesis 3). If TP causes extra gaps in nascent DNA via intercalation of DNA in front of the replication fork, then the chase time of these gaps should be the same with TP present during the chase as it is with TP absent. This explanation is not consistent with the observation that TP slows the chase of small pieces into control size DNA by 80%. If the pieces of DNA which appear during TP incubation are the result of TP stimulated general nucleolytic activity (hypothesis 3), then the

^{14}C-prelabeled DNA would not sediment to the control DNA position in the alkaline sucrose gradient. If TP stimulated the action of a "daughter-specific" nuclease (hypothesis 3), then nascent DNA pieces would not be expected to return to high molecular weight DNA (chase) in the presence of TP. Hypothesis 3 is not consistent with the observation that ^{14}C-prelabeled DNA is not degraded, and that pieces of DNA labeled in TP chase into high molecular weight DNA in the presence of TP. The observed results are most consistent with hypothesis 1: accumulation of nascent DNA pieces during the TP labeling period is caused by TP inhibition of some step(s) of normal replication gap closing.

Both recA and polA1 mutations have been shown to result in a special sensitivity to single strand breaks in their DNA (131), and the polA1 mutant crossed with a recA or recB mutant produce inviable progeny (227, 228). It has also been shown that polA1 cells close replication gaps at 10% of the rate that their pol$^+$ parent closes gaps (220, 229) even though replication proceeds at the same rate in both strains (57). However, a temperature sensitive polA1$_{ts}$ recA double mutant unexpectedly closes replication gaps at the same rate as the polA1$_{ts}$ single mutant when shifted to the nonpermissive temperature (215). Therefore, recA$^+$ gene product does not appear to be directly involved in gap closing (215). These observations

suggest that the lethality of the polAl⁻ condition for rec⁻ cells may be the result of the special sensitivity of the rec mutation to gaps in DNA. The introduction of the polAl⁻ gene into a rec recipient would be expected to increase the number of replication gaps 10 fold without any reduction in the rate of DNA synthesis (57, 220, 229), and as the rec mutation causes a special sensitivity to gaps in DNA, lethality results. The observation that recA gene product cannot directly contribute to the closure of replication gaps (215) suggests that the role of recA may be to maintain conditions which will allow the cell to remain viable in the absence of normal gap closing rates.

The interpretation that slower rates of replication gap repair are lethal for recA mutants correlates well with the effects reported here for TP. This photoproduct affects DNA replication by slowing the repair of replication gaps. Also, the photoproduct is selectively lethal for recombination deficient mutants and uncouples DNA replication from cell division in recA recB mutants. These observations suggest that the recA gene product plays a regulatory role in replication gap closing. Therefore, the following is proposed as a possible explanation of these results: the recA⁺ gene product senses the number of post-replication gaps by monitoring the configuration of the chromosome. When the number of

post-replication gaps increases, the recA$^+$ gene product

alters the replication complex to conform with the change

in chromosome configuration. In this way, DNA replication

would be coordinated with repair of post-replication gaps,

and when a stable chromosome configuration is reached

(i.e., the rate of appearance of post-replication gaps is

constant), DNA replication would resume a normal rate

in rec$^+$ cells. However, in the absence of functional

recA$^+$ gene product, DNA replication would fail to adjust

to any increase in the number of post-replication gaps,

and continuation synthesis would stop with the chromosome

and replication complex in an unrecoverable configuration.

This is consistent with the conclusion that the biological

specificity of TP for mutants deficient in recombination

repair is a result of the special sensitivity of these

mutants to gaps in their DNA. Therefore, TP kills log

phase rec mutants because the photoproduct slows the

rate of replication gap repair; this increases the number

of post-replication gaps, which is a lethal condition

for the cell.

B.   THE EFFECT OF TP ON DNA REPAIR

In addition to the effect of TP on DNA replication,

it also effects DNA repair. The following observations

indicate that TP is antagonistic for some step(s) involved

in the repair of gaps in DNA which result from far-UV

damage: (a) incubation (in TP) of wildtype cells exposed to a non-lethal dose of far-UV causes lethality at concentrations of TP which would not reduce the survival of unirradiated wildtype cells; (b) similar treatment of AB1884 uvrC also kills cells incubated in normally innocuous concentrations of TP; (c) similar treatment of AB1886 uvrA did not kill the treated cells.

Wildtype and uvrC cells have a functional "dimer-specific" endonuclease; uvrA lacks this endonucleolytic capability. This suggests that the effect of TP on excision repair (after UV damage) may be to interfere with attempts to repair the excision gap during the repair process. TP could antagonize the excision repair by inhibiting one or more of the following steps: (a) TP could inhibit the exonucleolytic removal of the damaged portion of the DNA; (b) TP could inhibit DNA synthesis in the excised portion of the strand; (c) TP could inhibit the rejoining of the resynthesized segment of DNA. If TP acts against one or more of these steps, postirradiation incubation would be more lethal for strains which can initiate excision (WT, uvrC) than for strains which cannot (uvrA).

The conclusion that TP acts against some step in gap repair is supported by the observation that incubation in TP enhances the lethal effects of X-irradiation of wildtype AB1157. Since the cells were exposed to X-

irradiation in buffer under aerobic conditions and then incubated in TP-M9 medium, essentially all of the "ultra-fast (ligase-mediated) repair" of X-ray damage should be complete before the TP incubation (131). Therefore, the enhancement of X-ray lethality observed for postirradiation incubation is not a result of action against the "ultra-fast repair" sector. The exposure of the cells to X-rays in aerobic conditions should cause damage to the DNA which will require both "excision-repair" and "recombina-tional-repair." Therefore, the TP enhancement of X-ray lethality could be the result of action against excision and/or recombinational repair.

The sedimentation profiles of DNA released from wildtype cells after exposure to X-irradiation and incuba-tion with and without TP present indicates that TP inhibits Type III (medium-dependent) repair of X-ray-induced single strand DNA breaks. This is based on the following observations: (a) WT cells exposed to 20 krad of X-irradiation, and incubated in M9 medium (no TP) for 30 minutes, closed 27 ss-breaks/ss-genome; (b) only 7 ss-breaks/ss-genome were closed in M9 medium with TP. This indicates that TP inhibits Type III repair of X-ray-induced single strand DNA breaks in the wildtype cells. In contrast, TP was only slightly inhibitory for polA1[+] mediated (Type II) repair of X-ray-induced single strand breaks in the DNA of WT cells. This is supported by the

observation that WT cells incubated in PBS buffer
following X-irradiation repaired 143 ss-breaks/ss-genome,
while cells incubated in PBS plus TP repaired 134 ss-
breaks/ss-genome. These results indicate that TP inhibits
Type II repair of X-ray-induced single strand breaks in
DNA by 6%. Therefore, the enhancement of X-ray lethality
in wildtype cells incubated in TP is probably the result
of the inhibition of some step(s) required to complete
Type III repair of X-ray induced single strand DNA breaks.
This inhibition of X-ray repair could involve a step(s)
shared by "medium-dependent" excision repair (85) and
recombinational repair of damaged DNA.

The sedimentation profiles of DNA released from
polA1 cells exposed to 16.9 krad of X-irradiation, and
then incubated with and without TP, demonstrates that
while TP strongly inhibits Type III repair in this strain,
the "slow" buffer (Type II) repair which occurs in the
polA1 mutant is relatively unaffected. The observation
of a "slow" buffer repair in polA1 strains incubated in
PBS at 37°C for extended periods is in good agreement
with repair observations reported by Town et al. (83) for
this mutant. However, Youngs and Smith have reported
that medium-dependent repair of far-UV damage to DNA
(rec^{+} dependent) requires the presence of functional DNA
polymerase III (85), and have further stated that DNA
polymerase III is required for "slow" buffer repair in

polAl mutants (83). Therefore, the observation that TP
does not substantially inhibit "slow" repair in the polAl
strain suggests that TP does not act against the DNA
polymerase III repolymerization step.

The sedimentation profiles in Figure 16 which show
the effect of TP on Type III repair in the polAl strain
indicate that TP inhibits medium-dependent repair by 94%
in this strain. Whereas the sedimentation profile of DNA
released from polAl cells exposed to X-ray and incubated
in M9 medium show the DNA degradation which occurs in
this strain (83), TP-M9 incubated polAl cells do not
demonstrate this degradation. This coinhibition of DNA
degradation could indicate that TP inhibition of medium-
dependent repair (Type III) is the result of blocking a
required "medium-dependent" nuclease. This interpreta-
tion is consistent with the observation that TP has only
a small inhibitory effect for either wildtype (polI-
mediated) buffer repair or "slow" buffer repair in a polAl
mutant. Therefore, these results indicate that TP is a
specific inhibitor of Type III repair of X-ray-induced
single strand breaks in DNA, and that Type II repair is
relatively unaffected. These data also suggest that the
inhibitory effect of TP may be the result of inhibition of
a nucleolytic function required to complete Type III
(but not Type II) repair of X-ray damage to DNA.

C.   INTERACTION OF TP AND MONOCHROMATIC

365 NM RADIATION

The results in Figure 17 indicate that the presence of TP in the suspension during 365 nm irradiation synergizes the rate of "alkali-labile bond" induction by 11.5 fold ($\frac{kTP}{kC}$). This 365 nm-TP interaction could be the result of one of the following: (a) TP sensitizes the DNA to 365 nm by photon absorption which results in photo-chemical interaction with the DNA (i.e., energy transfer, or adduct formation); (b) since TP inhibits repair in the dark, the extra DNA breaks could represent lesions left unrepaired in the presence of TP. However, since the 365 nm irradiation was conducted at $2^{\circ}C$, any enzymatic repair of damage to the DNA would be very slow. In addition, the observation that the exposure of the cells in Tris-EDTA buffer (an inhibitor of dark repair) does not alter the rate of induction of ss-breaks either in the presence or absence of TP, suggests that repair of these lesions does not occur during the irradiation. This leaves only the possibility that TP is inhibiting a repair mechanism which has not been described in the literature. Since this is not very likely, the increased rate of "alkali-labile bond" induction during 365 nm irradiation in TP probably does not involve the antagonism of DNA repair processes. It is therefore suggested that TP synergizes the induction of "alkali-labile bonds" during

365 nm irradiation by photosensitization of the DNA molecule.

The results in Figure 18 indicate that the presence of TP during irradiation enhances the lethal effect of 365 nm radiation by approximately 3 fold ($\frac{_c D_{37}}{_{TP} D_{37}}$) for polAl P3478. The D_{37} dose (1/e dose) is commonly used to represent the dose required for one lethal event in a treated population (171, 172). Although this rigorously applies only to populations which are killed exponentially (non-shouldered response), it is convenient for comparison purposes to use the D_{37} dose when a shouldered response is encountered, with the understanding that this is not an absolutely accurate description of events (171, 172). By comparing the number of TP-365 nm synergized breaks per D_{37} dose ($_{TP} D_{37}$ ss-breaks/genome) with the number of breaks induced by the D_{37} dose of 365 nm irradiation in buffer ($_c D_{37}$ ss-breaks/genome), the relative lethality of the TP-365 nm lesion can be estimated. The $_c D_{37}$ dose for polAl irradiated in M9 is 5.750 x 10^5 J/m^2, and the $_{TP} D_{37}$ dose is 1.825 x 10^5 J/m^2 (Figure 18). Calculation of the number of ss-breaks/genome which occur at the D_{37} dose in M9 shows that approximately 20 ss-breaks/genome is a lethal event for polAl, and a similar calculation indicates that approximately 67 TP-365 nm synergized ss-breaks/genome occur at the $_{TP} D_{37}$ dose. This calculation suggests that TP-365 nm synergized "alkali-labile bonds" in DNA are about

0.33 as lethal as the "alkali-labile bonds" in DNA which

occur in M9 buffer with no TP present.

This difference in lethality for TP-365 nm synerg-

ized ss-breaks in DNA vs. ss-breaks in DNA with no TP

present may be accounted for in several ways: (a) the

TP-365 nm DNA lesion is <u>different</u> <u>chemically</u> from the

lesion which occurs in the DNA when bacterial cells are

treated with 365 nm radiation without TP; (b) the lethal

effect of the ss-break (365 nm) is <u>dependent</u> on the

induction of another lesion (i.e., pyrimidine dimer),

which is present in much smaller numbers at the $_{TP}D_{37}$

requiring three times as many TP-365 nm synergized

"alkali-labile bonds" to cause a lethal event. The

observation that more TP-365 nm synergized DNA lesions

are required for lethality eliminates the possibility

that this treatment causes sensitized damage to the repair

systems. If that were the case, the TP-365 nm DNA lesions

would be <u>more</u> lethal than those which occur in the absence

of TP. A third possibility not mentioned above is that

the lethality of "alkali-labile bonds" is dependent on

365 nm inactivation of DNA repair systems. If 365 nm

inactivation of DNA repair plays a role in the lethality

of "alkali-labile bonds" then more TP-365 nm synergized

lesions would be required to saturate the repair systems.

If the TP-365 nm lesion is different from those which

occur in the absence of TP, then direct chemical analysis

of the DNA might be employed to separate the two lesions.
However, if a biological synergism involving more than one
lesion causes the observed difference in lethality,
experiments testing the effect of 254 nm dimers on TP-
365 nm "alkali-labile bonds" might provide some information
about this explanation.

D.   SOME CLOSING REMARKS ABOUT TP

The diagram in Figure 19 shows most of the infor-
mation available about the production of toxic photo-
products of L-tryptophan, the biochemical effects attri-
butable to TP, and the biophysical and biological con-
sequences of TP-Near-UV interaction. The action spectrum
for the production of toxic photoproducts of L-tryptophan
indicates that wavelengths shorter than 270 nm or longer
than 370 nm are very ineffective, and that 290 nm is the
peak of action for this effect (212). This action spectrum
suggests that more than one wavelength is involved in the
process since this action spectrum deviates significantly
from the molecular absorption spectrum of L-tryptophan
(212). Since the purging of a tryptophan solution before
and during near-UV irradiation with N_2 strongly inhibits
the production of biologically active TP, the photoreaction
may be concluded to have a requirement for oxygen and may
involve an excited singlet of molecular O_2 in the solution
(213). Inconclusive studies of the chemical properties

Figure 19.--Summarizing diagram indicating what has been determined at this time about the properties and modes of action of tryptophan photoproduct(s).

wavelengths
270nm-370nm

SUN ———— FLUORESCENT LIGHT

ξhv

H H
| |
—C—C—C
| | ‖
H NH$_2$ O
OH   (L-tryptophan)

TP

series of photooxidative
reactions

a stable, low molecular weight (250-500) molecule(s)
with at least one ring (~5-6 carbons)

PHOTOINTERACTION

synergistic
near-UV mutagenesis

synergistic
365 nm Inactivation

sensitizes bacterial
DNA to 365 nm induction
of "Alkali-labile" bonds

BIOCHEMICAL
ACTION

inhibits X-ray
UV-repair
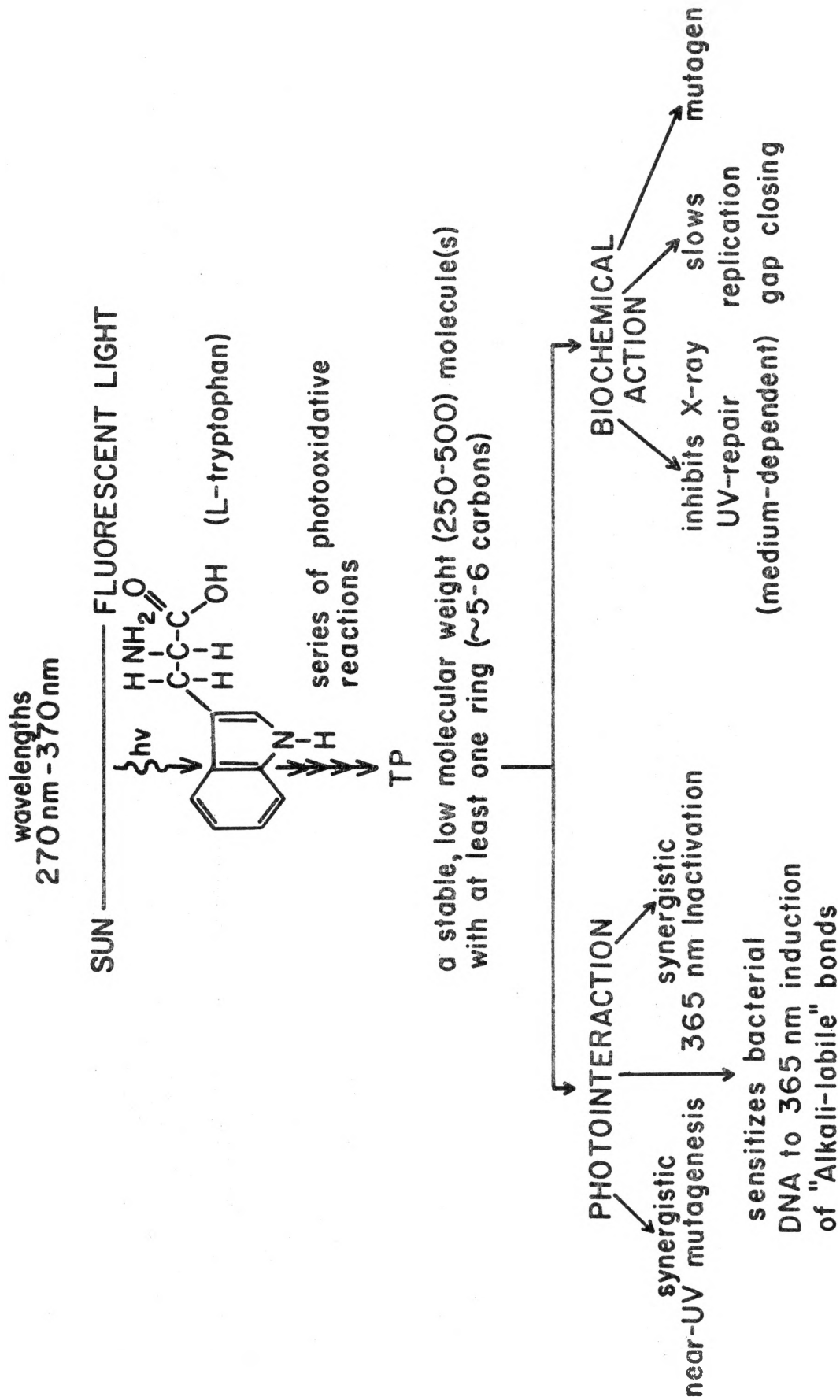(medium-dependent)

slows
replication
gap closing

mutagen

Figure 19

of TP have yielded the infomation that the active mole-
cule(s) in TP is a stable organic molecule (213), with
an approximate molecular weight of 250-500. Unfortunately,
we do not know the exact chemical structure. This infor-
mation when obtained will allow more detailed experimenta-
tion on the biological and biochemical effects of TP.

When bacterial cells are incubated in the dark in
medium containing TP, a number of biologically important
functions are affected. If the DNA of the cell has been
subjected to some treatment causing structural damage
to the DNA, TP blocks the medium-dependent repair of this
damage. If other processes of normal DNA metabolism, such
as DNA replication, introduce gaps into the DNA which
require medium-dependent repair to complete the chromosome,
then TP will also block this function. In fact, the
observation that TP inhibits the closure of replication
gaps in newly replicated DNA suggests that TP blockage of
medium-dependent repair of replication gaps might account
for the effect of TP on DNA replication. Moreover, it is
clear that TP does slow the closure of replication gaps,
and it is consistent with the data for the differential
sensitivity of DNA replication in polA1, and recA recB.
The effect of TP on replication gap closure may also
be involved in the dark mutagenicity of TP for E. coli
B/r/1 t⁻ when the cells are grown in turbidostat con-
tinuous culture in medium containing TP (212).

There are several near-UV - TP photointeractions which could be of some importance. Acute exposure of cells to broad spectrum near-UV in the presence of TP has not been tested for its effects on viability since (until recently) we did not have a light source with sufficient output. Also, the dosimetry was complicated. However, the effects of TP during chronic exposures of E. coli WP2 hcr to broad spectrum near-UV on mutation has been tested. The results of these experiments indicate that TP strongly synergizes the mutagenicity of chronic irradiation with broad spectrum near-UV (230). Since relatively high energy source of monochromatic 365 nm light is now available, and the dosimetry problems for the monochromatic irradiation of cells in solutions of TP are not difficult to overcome. The interaction of TP with monochromatic 365 nm radiation has been tested for "alkali-labile bond" induction and survival of polA1 P3478 cells. These results indicate that TP sensitizes cellular DNA to monochromatic 365 nm radiation causing a large increase in the yield of DNA lesions, and that these TP-365 nm induced lesions can account for the enhanced lethality observed for cells irradiated in TP.

VI. SUMMARY

Near-UV photoproduct(s) of L-tryptophan (TP) is
toxic for mutants of Salmonella typhimurium and Escherichia
coli deficient in the ability to carry out genetic recom-
bination (rec, exr). This study provides evidence that
the toxic effect of TP for rec mutants is the result of
inhibition of a step(s) required for the closure of gaps
in DNA. These gaps occur during normal chromosome
replication, as well as upon structural damage to the
DNA molecule. The observation that the 7×10^6 dalton
pieces of DNA in growing wildtype cells are followed by
a slow "chase" of these replicative intermediates in the
presence of TP, indicates that TP inhibits the rapid
closure of replication gaps. Alkaline sucrose gradient
analysis of DNA released from X-ray treated wildtype
cells, and incubated to allow repair in the presence
and absence of TP, shows that TP inhibits buffer repair
(Type II) by 6%, and medium-dependent (Type III) repair
by 75%. These different types of repair have been
previously defined, based upon "post-damage" conditions,
usually the medium in which the cells are held before
plating. These experiments demonstrate that TP inhibits
a particular type of gap closure process (Medium-Dependent)
which may be essential for survival in rec mutants. The

effect of TP on the repair of X-ray induced breaks in DNA and the closure of gaps which result during DNA replication is consistent with the interpretation that the rec^+ repair system interacts with the replication complex to "stablize" gaps; this allows repair and replication to occur in a coordinated manner.

The effect of TP on the rate of DNA replication in wildtype, uvrA, polA1, and recA recB strains of E. coli was tested by measuring the rate of incorporation of ^{3}H-thymidine into TCA insoluble radioactive DNA in the presence and absence of TP. The effect of TP on cell division was also ascertained; this was done by measuring the increase in cell numbers and the size distribution with a Coulter counter during incubation of the cells in TP-M9 medium. The results of these experiments indicate that TP differentially inhibits DNA replication in the polA1 mutant and the recA recB double mutant; wildtype, and uvrA recover normal replication rates during the TP-M9 medium incubation. Thus, the effect of TP on DNA replication suggests that mutants deficient in gap repair functions (polA1 and recA recB) are more sensitive to TP than other strains. This parallels the observation that TP uncouples DNA replication from cell division only in the recA recB strain. The uncoupling conclusion is supported by the observation that, while DNA replication is completely inhibited in both recA recB and polA1 strains,

cell division stops only in the polA1 mutant; the recA recB strain continues to divide during the TP-M9 medium incubation. These results indicate that the effect of TP on gap repair processes in the rec mutant may be to uncouple DNA replication from cell division. The ultimate result of this uncoupling is cell death.

TP was also tested for its synergistic interaction with monochromatic 365 nm radiation for the production of DNA lesions, detected with the alkaline sucrose gradient technique. Also, polA1 stationary phase cells were tested for enhanced sensitivity to 365 nm radiation when cells were irradiated while suspended in buffered TP. The results of these experiments show that TP sensitizes bacterial DNA to 365 nm induction of "alkali-labile bonds", and that these DNA lesions contribute to the inactivation of polA1 when these cells are irradiated in the presence of TP. The alkaline sucrose gradient data indicate that TP increases the rate of "alkali-labile bond" production by 11.5 fold, and enhances the killing of polA1 cells irradiated in TP by 3-fold. These data demonstrate that TP sensitizes bacterial DNA to 365 nm radiation, and that the lesions produced by this treatment contribute to the inactivation of polA1 cells irradiated in TP-M9 solutions. These results suggest that TP (most efficiently produced by 290 nm photolysis of tryptophan) may play a role in synergistic effects which involve the interaction of mid-UV (280-320 nm) and near-UV (320-405 nm) radiation.

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