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# UV INACTIVATION AND THYMINE DIMERIZATION IN BACTERIOPHAGE $\Phi$X

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With 3 Figures in the Text

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## Introduction

Measurements of the UV sensitivity of $\Phi$X DNA—both single-stranded (ss) and double-stranded (replicative form-RF)—indicate that the biological activity of the RF is only 1/10 as sensitive to UV as that of ssDNA (Fig. 1). Recently JANSZ, POUWELS and VAN ROTTERDAM (1963), YARUS and SINSHEIMER (1964) and SAUERBIER (1964) have shown this decreased sensitivity of the double-stranded form to be the result of a type of biological repair of UV lesions (host cell reactivation—hcr) which can take place in the RF but not in the ssDNA. The repair mechanism is a bacterial function and can be lost by bacterial mutation ($hcr^+ \rightarrow hcr^-$).

A formally similar situation exists when bacteriophage T4$v_1$ (containing double-stranded DNA) is compared to $\Phi$X. T4$v_1$ has 75 times more DNA and might be expected to be 75 times more sensitive to UV. However, measurements show it to be only 5 times more sensitive than $\Phi$X to UV irradiation (Fig. 1). This discrepancy between DNA content and UV sensitivity has not been shown to be the result of an enzymatic repair mechanism in T4. No bacterial or phage mutations have been found greatly increasing the UV sensitivity of T4$v_1$, although one mutant of T4 is known — T4$v_1$x — with a UV sensitivity about twice that of T4$v_1$ (HARM, 1963).

Besides the biological reactivation mentioned above, there exists a second possible explanation for the discrepancy between the DNA contents and UV sensitivities of these two phages — namely a difference in the photochemical reactivity of single-stranded and double-stranded DNA. Our knowledge of the chemical nature of UV photoproducts in DNA is too incomplete to test this explanation in full generality. However, thymine dimerization does occur in UV'd DNA and thymine dimer ($\widehat{TT}$) has been suspected of being a UV lethal lesion. (BEUKERS, IJLSTRA and BERENDS, 1960; WACKER, DELLWEG and WEINBLUM, 1960). If thymine dimers do contribute to lethality in DNA, a comparison of quantum yields for dimerization in the two phages could tell whether or not the discrepancy is a result of differences in photochemical reactivity. The quantum yield for thymine dimerization in T4$v_1$ has already been measured by WULFF (1963a). We have used his methods to measure the quantum yield for $\widehat{TT}$ formation in $\Phi$X. We find that the two quantum yields are very similar.

The question of the lethality of $\widehat{TT}$ in DNA is not settled. If every $\widehat{TT}$ represents an irreversible (not biologically repairable) lethal lesion in DNA of bacteriophage, the number of $\widehat{TT}$ per phage lethal hit must be $\leqq 1.0$. WULFF's (1963a) measurement of 4.8 $\widehat{TT}$/hit in T4v$_1$ proves that in this phage *not* every $\widehat{TT}$ is irreversibly lethal, perhaps because of the biological repair mentioned above. We find 0.34 $\widehat{TT}$/hit in $\Phi$X. This result proves the existence of *other lethal photoproducts* in $\Phi$X DNA produced with an efficiency comparable to thymine dimerization.

## Materials and Methods

*Bacteria and Bacteriophage.* The bacteriophage $\Phi$X174 (obtained from Dr. R. L. SINSHEIMER) was used in these studies. The host bacteria were *E. coli* C and CR34-C416, a thymine requiring mutant (also obtained from Dr. SINSHEIMER).

*Media:* Tryptone Broth: Bacto-Trytone (Difco) 10 g; NaCl 5 g; 1 M-NaOH 1 ml; $H_2O$ 1 liter; M9: $Na_2HPO_4 \cdot 7H_2O$ 7.0 g; $KH_2PO_4$ 3.0 g; $NH_4Cl$ 1.0 g; $H_2O$ 1 liter.

K Medium: 2X M9 50 ml; 3% Casamino acids (Difco) purified with charcoal 50 ml; 25% NaCl 0.2 ml; 1.0 M-$MgSO_4$ 0.25 ml; 10% Glucose (w/v) 2.0 ml.

DNase and RNase were obtained from the Worthington Biochemical Corp.

Methyl-[$^3$H]-thymine for labelling phage stocks was obtained from the New England Nuclear Corp. with a specific activity of 9.2 c/mM.

[$^{14}$C]-thymine dimer was prepared by irradiating a frozen solution of methyl-[$^{14}$C]-thymine (New England Nuclear Corp.) with a germicidal lamp and purifying the dimer by paper chromatography in butanol: water (86:14).

*Plating.* Virus assay was performed by the agar layer method (ADAMS, 1959). Bottom agar and top agar were those of SINSHEIMER (1959). UV survival curves were plated in dim yellow light to prevent photoreactivation.

*Growth and Purification of the [$^3$H] thymine $\Phi$X 174. E. coli* CR34-C416 was grown overnight in K medium plus 10 $\mu$g/ml thymidine. The overnight culture was diluted 1/100 into fresh K medium (250 ml) plus 10 $\mu$g/ml thymidine and grown at 37° C to 1—2 $\times 10^8$ cells/ml. The cells were collected by filtration on a large size 0.45 $\mu$ HA millepore filter (Millepore Filter Co., Bedford, Mass.), washed with 1—2 volumes of M9, resuspended from the filter pad in fresh K medium (250 ml) and bubbled at 37° C to use up any thymidine not washed out on the filter pad. After 5 minutes, [$^3$H]-thymine 2 $\mu$g/ml (230 mc/mM) and $\Phi$X174 (m.o.i. = 3) were added. Forty-five minutes after infection, 2.5 ml 0.2 M-EDTA at pH 7.0 was added to minimize the readsorption of virus particles onto bacterial debris. Sixty minutes after infection 1 $\mu$g/ml DNase and 1 $\mu$g/ml RNase were added and incubation continued for 15 minutes at which time the lysate had cleared.

The purification of $\Phi$X174 particles from the crude lysate was accomplished by the procedure of HALL and SINSHEIMER (1963) except for the DEAE-cellulose adsorption step. A final solution of purified phage (6.5 ml in M9) was obtained having $1.2 \times 10^5$ cpm/ml and a total of $5 \times 10^{12}$ plaque-forming particles.

*Ultraviolet Irradiation.* Irradiation was carried out at 260 m$\mu$ in a water prism monochromator (JOHNS and DELBRÜCK, unpublished)[1]. The use of the instrument, procedures for calibration and dose calculations, including corrections for volume changes and solutions having significant absorbancy, have been described by WULFF (1963a).

*Inactivation.* Samples of labeled phage (3 ml) were irradiated in the monochromator. Fig. 1 shows the typical single-hit inactivation curve observed. The effective dose required to inactivate to a survival of $e^{-1}$ (one hit) is about 65 ergs/mm$^2$. For samples irradiated to

[1] The experiment was performed twice: once in Cologne, Germany, on the water prism monochromator and once in Pasadena, California, on a double quartz prism monochromator (WINKLER, JOHNS, and KELLENBERGER, 1962). A small difference exists between the calibrations of these two instruments. All data in the body of the manuscript refers to the Pasadena instrument. The Table summarizes results from both experiments.

high doses (25, 50, 100 hits) the inactivation curves were measured only for the first 10 hits ($5 \times 10^{-5}$ survival). Six such curves were obtained having slopes ranging from 65.1 to 72.2 ergs/mm$^2$ for a $e^{-1}$ decrease in the fraction of surviving phage (average 67.9 ergs/mm$^2$).

*Analysis for [$^3$H]-thymine and [$^3$H]-thymine dimer:* The phage solution remaining in the cuvettes following irradiation and sampling for the inactivation measurements (usually about 2.8 ml) was dialyzed in the cold against 3 changes of distilled water (18 hrs).

Following dialysis each sample was divided into 3 heavy-wall pyrex tubes for hydrolysis. The tubes were evaporated slowly to dryness in a hot water bath under a gentle stream of air. When evaporation was complete, the tubes were cooled and 0.5 ml 88% formic acid was added to each. Hydrolysis was carried out for 30 minutes at 175° C in evacuated sealed tubes. (WYATT and COHEN, 1953). The hydrolyzed samples were transferred to strips of Whatman No. 1 paper. Suitable quantities of [$^{14}$C]-thymine dimer were added to each strip as controls to permit subsequent correction for chromatographic losses.

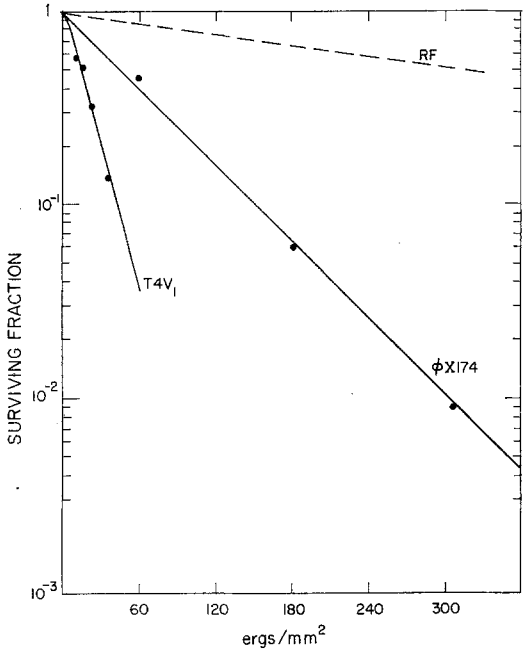


Fig. 1. Typical UV inactivation curves for ΦX and T4v$_1$ under conditions of no photoreactivation. One hit doses are given in the Table. The curve for RF in hcr$^+$ bacteria is taken from SINSHEIMER *et al.*, (1962)

Chromatography was done in butanol: water (86:14) to separate [$^3$H]-thymine and [$^3$H]-thymine dimer. The dimer was further purified by two more chromatographies in butanol: water, converted to thymine by UV irradiation, followed by chromatography in butanol: water and a final chromatography in saturated ammonium sulfate: sodium acetate (1 M): isopropanol (40:9:1). Details of the chromatographic procedure are described fully by WULFF (1963a).

The spots containing thymine and thymine dimer were cut out and placed in polyethylene vials for liquid scintillation counting. One ml of water was added to each vial followed 30 minutes later by 15 ml of dioxane-scintillator solution (recrystallized naphthalene 150 g; PPO [Packard Instruments] 7 g; POPOP [Packard Instruments] 0.375 g; dioxane 1 liter). The salts and paper from chromatography did not affect the counting. Using double channel counting on a Packard liquid scintillation spectrometer, the samples were analyzed for $^3$H and $^{14}$C.

The processing of the data has been described by WULFF (1963a).

The accuracy of the dimer determination is quite high. Any loss during chromatography is corrected for by the [$^{14}$C]-thymine dimer added as a control immediately following hydrolysis. Losses in hydrolysis cannot be checked directly. However, several experiments indicate that the recovery of dimer after hydrolysis is quantitative. (1) WULFF (1962) found the yield of thymine and thymine dimer from irradiated DNA to be independent of hydrolysis time over a range of 15 to 60 minutes at 175° C. (2) WACKER and TRÄGER (1963) have shown that the saturation of the 5,6 double bond of thymine in DNA by dimerization with another thymine greatly weakens the N-glycosidic linkage and thus facilitates acid hydrolysis.

## Results

*Rate of Dimerization.* The fraction of thymine present as dimer for one experiment has been plotted in Fig. 2 and 3 as a function of the effective UV dose.

The increase is linear (up to 6000 ergs/mm$^{-2}$) with a slope of $5.56 \times 10^{-6}$ fraction thymine present as dimer per erg/mm$^2$ or $4.24 \times 10^{-19}$ fraction thymine present as dimer per quantum/cm$^2$. Taking the extinction coefficient for $\Phi$X174 DNA as

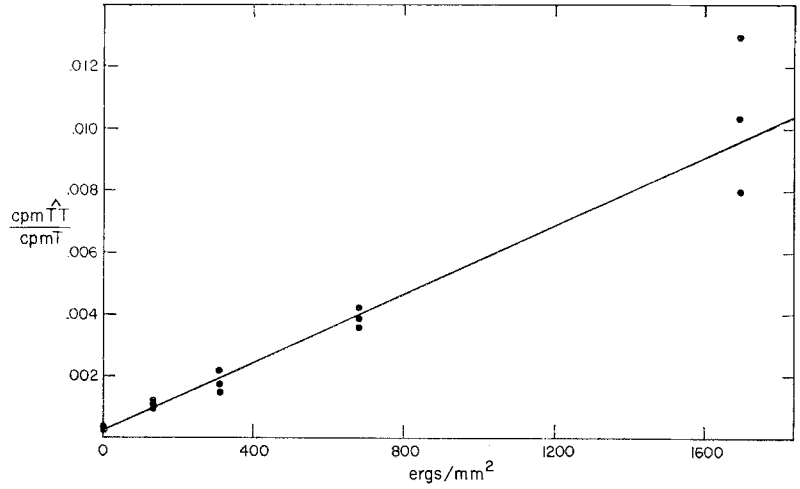


Fig. 2. Fraction of thymine present as dimer in $\Phi$X174 versus dose at 260 m$\mu$

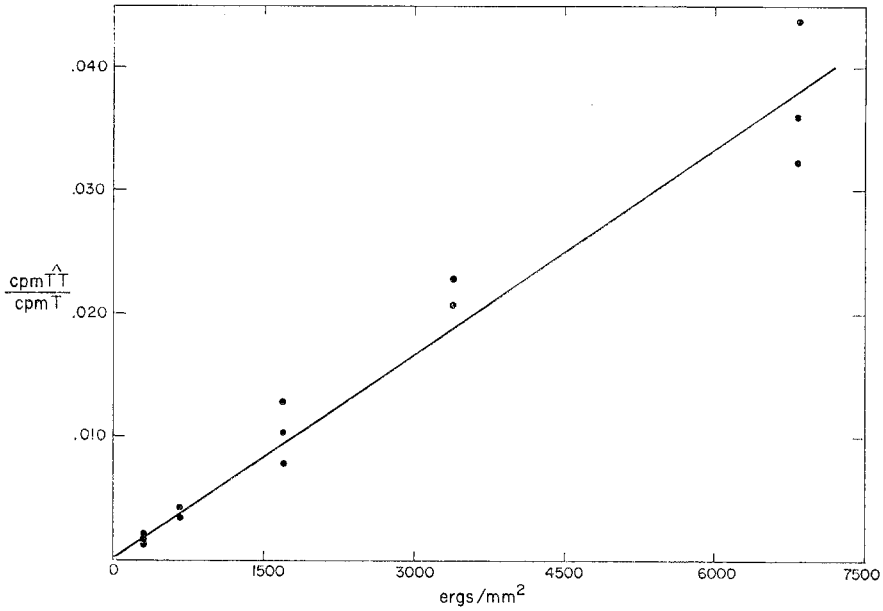


Fig. 3. Fraction of thymine present as dimer in $\Phi$X174 versus dose at 260 m$\mu$. The slope is $5.56 \times 10^{-6}$ cpm $\hat{TT}$/cpm T per erg/mm$^2$

8500 per P atom (Sinsheimer, 1959) the absorption cross section is $3.23 \times 10^{-17}$ cm$^2$ and the quantum yield 0.013 fraction thymine present as dimer per quantum absorbed by a nucleotide. This is very close to values of 0.019 for T4 $v_1$ (Wulff, 1963a) and 0.013 for E. coli DNA (Wulff, 1963b). Values for two independent experiments are tabulated in the Table.

Table. *Rates of thymine dimerization, quantum yields and $\widehat{TT}$/hit calculations for $\Phi X$ and $T4v_1$* (WULFF, 1963a)

Experiment I and WULFF's data were obtained on the same water prism monochromator; Experiment II was done on a quartz prism monochromator. The differences in the results between I and II are principally the result of a consistent difference in the calibrations of the two instruments. (See Footnote in Materials and Methods).

The slopes of the cpm $\widehat{TT}$/cpm T versus erg/mm$^2$ plots (Fig. 2 and 3) have been multiplied by $^1/_2$ to convert to molar ratios: cpm $\widehat{TT}$/cpm T $= 2[\widehat{TT}]/[T]$.

| | $\Phi$X174 | | T4 | Units |
|---|---|---|---|---|
| | I | II | (WULFF, 1963a) | |
| Rate of thymine dimerization | $3.84 \times 10^{-6}$ | $2.78 \times 10^{-6}$ | $3.05 \times 10^{-6}$ | $\frac{[\widehat{TT}]/[T]}{erg/mm^2}$ |
| UV dose to inactive to $e^{-1}$ survival . . . . . | 58 | 67.9 | 12 | ergs mm$^{-2}$ |
| Qantum yield . . . . . | .018 | .013 | .019 | 2 $[\widehat{TT}]/[T]$ per quantum absorbed by one nucleotide |
| $\widehat{TT}$/hit . . . . . | .398 | .338 | 4.8 | |

The discovery of trinucleotides of the form, pXp$\widehat{TpT}$, in enzymatic digests of irradiated DNA (BOLLUM and SETLOW, 1962; SETLOW, CARRIER and BOLLUM, 1964) and the low quantum yield for cross-linking in UV irradiated DNA (FREIFELDER and DAVISON, 1963) make it highly likely that thymine dimers in irradiated DNA are formed between adjacent thymines in one polynucleotide chain. Therefore, the quantum yield for thymine dimerization should depend on the frequency of pTpT sequences in DNA — i.e. the thymine-thymine nearest neighbour frequency. $\Phi$X and T4 have similar thymine-thymine nearest neighbour frequencies: 0.099 and 0.109 respectively (JOSSE *et al.* 1961; SWARTZ *et al.* 1962). Thus the quantum yields for thymine dimerization in these two bacteriophages may be validly compared. The pTpT frequency for *E. coli* DNA is 0.08.

*Thymine Dimers per lethal hit.* If every thymine dimer is an irreversible lethal hit in bacteriophage, the number of dimers per lethal hit is constrained by the equation $\widehat{TT}$/hit $\leqq 1.0$. (WULFF has pointed out that this figure might be as high as two for phages having inactivation curves which do not follow single hit kinetics but rather some approximation to two hit or two target kinetics.)

The number of dimers per lethal hit is calculated as the product of three factors: 1790 thymines per $\Phi$X174 DNA molecule (HALL and SINSHEIMER, 1963); $2.78 \times 10^{-6}$ $[\widehat{TT}]/[T]$ per erg/mm$^2$ — the rate of dimerization (Table); and 67.9 erg/mm$^2$ — the UV dose required to inactivate $\Phi$X174 to $e^{-1}$ of the original titer (Table). The result is 0.34 $\widehat{TT}$/hit (Table).

## Discussion

We find similar quantum yields for $\widehat{TT}$ formation in the phages T4v$_1$ and $\Phi$X. This close similarity between a phage containing single-stranded DNA ($\Phi$X) and

one containing double-stranded DNA ($T4v_1$) should be contrasted with the finding of WACKER *et al.* (1962) that the quantum yield for thymine dimerization in denatured *E. coli* DNA was about twice that for native DNA. SETLOW, CARRIER, and BOLLUM (1964) have observed qualitatively the same result as WACKER *et al.* — namely the occurrence of more trinucleotides of the general form $pXp\widehat{TpT}$ in enzymatic digests of denatured DNA than in native DNA irradiated with the same dose of UV (X can be any of the four bases). Our results could reflect some kind of ordered packing of the DNA inside the ΦX particle more comparable to native DNA than to denatured DNA in solution.

Until now the only UV photoproduct isolated from DNA has been thymine dimer. Considerable effort has been applied in attempts to show that it is a lethal UV lesion (WULFF, 1963a; 1963b). Our measurement of 0.34 $\widehat{TT}$ per phage lethal hit demonstrates the existence of *other lethal photoproducts*, at least in ΦX DNA. It is thus worthwhile reviewing briefly what is known about thymine dimerization and seeing whether our findings of other lethal photoproducts could explain why the UV sensitivity of $T4v_1$ is lower relative to its DNA content than is the UV sensitivity of ΦX.

This discrepancy between DNA contents and UV sensitivities in $T4v_1$ and ΦX can not be due to the single-stranded nature of ΦX DNA. This can be seen by noting that RF (the double-stranded form of ΦX) when plated under conditions of no biological reactivation (on $hcr^-$ bacteria), has a UV sensitivity about 8X less than $T4v_1$ although it contains about 40X less DNA. (YARUS and SINSHEIMER, 1964). Furthermore, we have shown that this discrepancy can not be due to differences in the quantum yield for thymine dimerization. Therefore, if thymine dimers are lethal, they must be subject to some kind of biological repair or bypass in $T4v_1$.

Thymine dimers have, in fact, been shown to be affected by two biological reactivation systems. WULFF and RUPERT (1962) reported the loss of $\widehat{TT}$ from UV'd DNA following *in vitro* treatment with a crude preparation of the photoreactivation enzyme. Recently BOYCE and HOWARD-FLANDERS (1964) have shown that $\widehat{TT}$ is one of two thymine-containing photoproducts excised from DNA by the hcr enzyme system. However, when a direct search was made for alterations in $\widehat{TT}$ following infection of bacteria by UV'd T4, none were found. SAUERBIER tested for both excision and monomerization of $\widehat{TT}$ and found neither[1]. SAUERBIER's results indicate that thymine dimers in UV'd T4 probably remain in the DNA during replication which suggest that they are not lethal.

Lethal photoproducts, other than $\widehat{TT}$, necessary to explain ΦX inactivation, offer an alternative explanation for the discrepancy between the DNA contents and UV sensitivities of $T4v_1$ and ΦX. According to our measurements these photoproducts must constitute at least 60% of the lethal lesions in ΦX. If these lesions, which are not repaired in ΦX, occurred in T4 DNA and were subject to reactivation mechanisms in this phage, the high resistance of $T4v_1$ to UV could be explained.

[1] Bioch. Biophys. Acta, in press.

## Summary

Thymine dimers are found in DNA following irradiation at 260 mμ. The quantum yield for dimer formation in bacteriophage ΦX (single-stranded DNA) is 0.013 dimers per quantum absorbed by a nucleotide. This is comparable to the quantum yield for bacteriophage $T4v_1$ (double-stranded DNA) indicating there is no dependence of thymine dimerization on the nature of the irradiated DNA.

In ΦX the number of thymine dimers per lethal hit is 0.34. This demonstrates the existence of other as yet unidentified lethal photoproducts in irradiated ΦX DNA.

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