[9] Wilson, E. B., *J. Amer. Statist. Assoc.*, **22**, 209–212 (1927). Mr. Neyman, on page 222 of his *Lectures and Conferences on Mathematical Statistics and Probability* (USDA, 1952), suggested that I then had the idea of confidence intervals. I would make no such claim. I was merely trying to say that for logical reasons statisticians should treat an unknown probability as unknown and in using a standard deviation should allow for any Lexian ratio that rates of the sort they were considering might be expected to show.

[10] As a result of the sameness of the root-mean-square, the "critical ratio" for any value of V common to the two must be the same, but the probabilities will usually be different. Thus, for $m = 2, n = 2$ and for $m = 3, n = 1$ with $p = 2/3$, the critical ratio will be 2.0, for $V = \pm 1$, but the probabilities will be, respectively, 0.10 and 0.12 for us, 0.32 and 0.50 by chi-square with the Yates correction, and 0.17 and 0.25 for Fisher with his one-sided approach, and I daresay twice that for Yates according to his original paper in *Supp. J. Roy. Statist. Soc.*, 1, 217 (1934). I agree with Yates for the symmetrical case, but consider the asymmetrical case not yet decided [see my note in these PROCEEDINGS, **39**, 537–546 (1953)]. It looks as though, if an experimenter desires to increase the probability of the values of V, he should take m and n as nearly alike as he can; if he is only interested in the "error to be feared," it does not matter.

[11] The detailed results like those in Table 1 are routinely obtainable. For $N = 6$, the respective probabilities are for $V = \pm 1$:

$$2x^3 \text{ for } m = 3, n = 3;\ x^2 - 2x^3 \text{ for } m = 4, n = 2;\ x - 4x^2 + 2x^3 \text{ for } m = 5, n = 1.$$

The first two maximize at 1/32 when $x = 1/4$; the third maximizes when $x = 0.13962$ at 0.0671, more than twice its value when $x = 1/4$. For the case of association the corresponding probabilities are

$$20\,x^3y^3,\ 15\,(x^2 - 2x^3)\,(y^2 - 2y^3),\ 6(x - 4x^2 + 2x^3)\,(y - 4y^2 + 2y^3).$$

The first two maximize when $x = y = 1/4$ at 0.00967 and 0.014165, whereas the last does when $x = y = 0.13962$ at 0.02814. Thus, no one of the perfect associations is likely to be observed. As the maxima occur at different values of $x = y$, we may not add them but must add the probabilities and determine the nature of that total probability, which is:

$$124x^3y^3 - 78x^2y^3 - 78x^3y^2 + 111x^2y^2 + 12x^3y + 12xy^3 - 24x^2y - 24xy^2 + 6xy.$$

For $x = y$ the value of this probability will be 0.0303 when x is 1/4, 0.0288 when x is 0.228, 0.0312 when x is 0.164, and remains within a range of about 0.0030 for the values of $x = y$ from 1/8 to 1/4, i.e., of p or r from 0.15 to 0.85, provided they are equal or complementary.

RELEASE OF ULTRAVIOLET LIGHT-INDUCED THYMINE DIMERS FROM DNA IN E. COLI K-12

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Communicated by Max Delbrück, January 2, 1964

Many lines of evidence support the concept of dark reactivation of ultraviolet (UV)-induced damage in bacterial and phage deoxyribonucleic acid (DNA).[1–6] In a previous paper we reported genetic crosses between a UV-sensitive F^- strain of *E. coli* K-12 (AB1886) and several Hfr strains.[7] The results of such crosses suggest that the reactivation system, presumably enzymatic in nature, is controlled by genetic loci which can be identified on the Hfr chromosome. A locus *uvr*A,[8] is defective in the UV-sensitive mutant, AB1886, with the result that the reactivation system is nonfunctional in this strain. It was found that UV-irradiated phage which contained 5-bromodeoxyuridine in place of thymine in DNA

were not reactivated. This result suggests that some photoproducts associated with thymine are involved in the reactivation of DNA. Moreover, reactivation in the resistant strain or photoreactivation in the sensitive strain can render harmless about 80% of otherwise lethal UV photoproducts in the phage DNA. Since a photoreactivation enzyme system from yeasts splits thymine dimers[9] ($\widehat{TT}$) in transforming DNA,[10] reactivation in the resistant strain may also involve thymine dimers. Setlow *et al.*[11] showed that in *E. coli* B/r thymine dimers are conserved in whole cells after UV irradiation. In the present paper we report evidence for the removal of thymine dimers from DNA in strain *E. coli* K-12 AB1157 and that such a removal does not occur in a UV-sensitive mutant strain, AB1886, derived from the resistant strain. This is shown as a decrease in the dimer/thymine ratio in the acid-insoluble fraction of thymidine-methyl-H^3 (H^3-TdR) labeled bacteria which had been incubated after UV irradiation, and the concomitant appearance of thymine dimer in the acid-soluble fraction. This change did not occur in the sensitive nonreactivating mutant, AB1886. Setlow and Carrier[12] have shown that a similar excision of thymine dimer occurs in strain B/r, but not in B_{S-1}, a UV-sensitive mutant of strain B isolated by Hill.[13]

Materials and Methods.—Bacterial strains as previously described[7] were derived from *E. coli* K-12 and are

AB1157 F^- *thr leu pro his thi arg lac gal ara xyl mtl* $T1^S$ $T6^R$ λ^S str^B
AB1886 F^- *thr leu pro his thi arg lac gal ara xyl mtl* $T1^S$ $T6^R$ λ^S str^R *uvrA*-6^R.

The abbreviations used are: F^- = genetic recipient; metabolic requirements are indicated by *thr* = threonine; *leu* = leucine; *pro* = proline; *his* = histidine; *thi* = thiamine; *arg* = arginine; inability to utilize carbohydrates is indicated by *lac* = lactose; *gal* = galactose; *ara* = arabinose; *xyl* = xylose; *mtl* = manitol; $T1^S$ = T1 phage-sensitive; $T6^R$ = T6 phage-resistant; λ^S = lambda phage-sensitive; str^R = streptomycin-resistant; *uvr* = inability to reactivate UV-irradiated DNA.

Labeling of DNA: The DNA of the bacteria was labeled with H^3-thymidine by growth in M9 medium[14] which contained 2.5 mg/ml vitamin-free casamino acids, 0.5 μg/ml thiamine, 250 μg/ml deoxyadenosine,[15] and 0.5 μg/ml H^3-TdR. The latter from New England Nuclear had a specific activity of 17.1 C/mM. After reaching early stationary phase, cells were washed five times by centrifugation and suspended in M9 medium for irradiation.

Irradiation: Thirty ml of M9 containing 5×10^7 cells per ml were irradiated in 14-cm diameter Petri dishes on a rotary shaker with a low-pressure mercury germicidal lamp. The suspensions were 55–65% transparent to the 2537 A light. The dose rate was 10 ergs/mm^2 sec as determined by a General Electric germicidal UV meter. The average dose to the cells, unless otherwise noted, was 960 ergs/mm^2 which resulted in a survival of 10^{-1} for the resistant AB1157 cells and 10^{-6} for the sensitive AB1886 cells.

Postirradiation treatment of cells: After irradiation, the cells were either chilled in ice water or incubated in the dark at 37°C in M9 with or without 0.4 mg/ml casamino acids and 0.1 μg/ml thiamine. This supplemented M9 is called EM9. Deoxyadenosine was not present in either medium. Resistant cells which had received a dose of 960 ergs/mm^2 would double in number if incubated two hours in EM9 before the medium became deficient in an essential nutrient. The growth of these cells was inhibited in M9. The irradiated sensitive cells did not grow in either medium.

Preparation of cells for hydrolysis: Treated cells were centrifuged, washed, suspended in 1 ml of cold 5% trichloroacetic acid, and left at ice-water temperature for 45 min. The acid-soluble fraction was decanted after centrifugation. The acid-insoluble fraction was washed in 0.5 ml of cold acid, and after centrifugation the supernatants were combined and dried.

Hydrolysis: Hydrolysis was carried out for 90 min at 170–175°C in sealed, evacuated glass tubes containing 1.0 ml of concentrated trifluoroacetic acid and 3–6 $\times$ 10^9 cells. Under these conditions no degradation of reference thymine dimer could be detected. After cooling, the tubes

were opened and the contents dried. The final residue was suspended in 0.04 ml 0.1 *N* HCl.

Chromatography: The hydrolysates were applied to Whatman #1 paper strips and descending chromatography was carried out at room temperature in n-butanol/acetic acid/water (200:30:75). The strips were dried at room temperature and scanned for H^3 activity in a 4π gas-flow chromatogram scanner which had a counting efficiency for H^3 of 1%.

Thymine dimer: Thymine dimer[9] used as a reference in this experiment was produced by irradiation of a frozen aqueous solution containing 1 mg/ml thymine and 10 μC/ml C^{14}-thymine with an estimated dose of 10^6 ergs/mm^2. This resulted in a 70% decrease in optical density at 265 mμ. Chromatography of the products indicated the presence of two major components (see Fig. 3*a*). One had thymine, which in the n-butanol/acetic/acid/water solvent system had a R_f value of 0.59 $\pm$ 0.2; the other, presumably thymine dimer, had an R_f value of 0.28 $\pm$ 0.1. No further purification was carried out. Thymine dimer thus produced is called reference thymine dimer. Thymine dimer among the photoproducts of irradiated DNA was identified by its R_f value, and by its reversion to thymine after UV irradiation in solution. Alleged dimer was eluted from the paper strips in 2–3 ml water and irradiated with an estimated dose of 10^5 ergs/mm^2. Under these conditions, conversion of reference dimer was nearly quantitative (see Fig. 3*a* and 3*b*).

Plan of the experiment: Cells labeled in their DNA with H^3-TdR were divided into 4 samples. One sample was extracted with cold trichloroacetic acid without UV irradiation. The remaining three samples were irradiated, and one sample was immediately chilled, centrifuged in the cold, and treated with cold acid. The remaining two samples were incubated in EM9 and in M9 for two hr in the dark at 37°C before centrifugation and extraction with cold acid. Both soluble and insoluble fractions were hydrolyzed in hot trifluoroacetic acid, except as otherwise noted, and the products separated by paper chromatography.

Results.—Figure 1 shows typical radiochromatograms of hydrolyzed acid-insoluble fractions of cells unirradiated, or irradiated with or without subsequent incubation in the dark. Two photoproducts, indicated by arrows in Figure 1*c*, appeared after irradiation without subsequent incubation. The photoproduct represented by the larger of two peaks is thought to be thymine dimer as judged by its R_f value of 0.27, and by its reversion to thymine when eluted and irradiated,

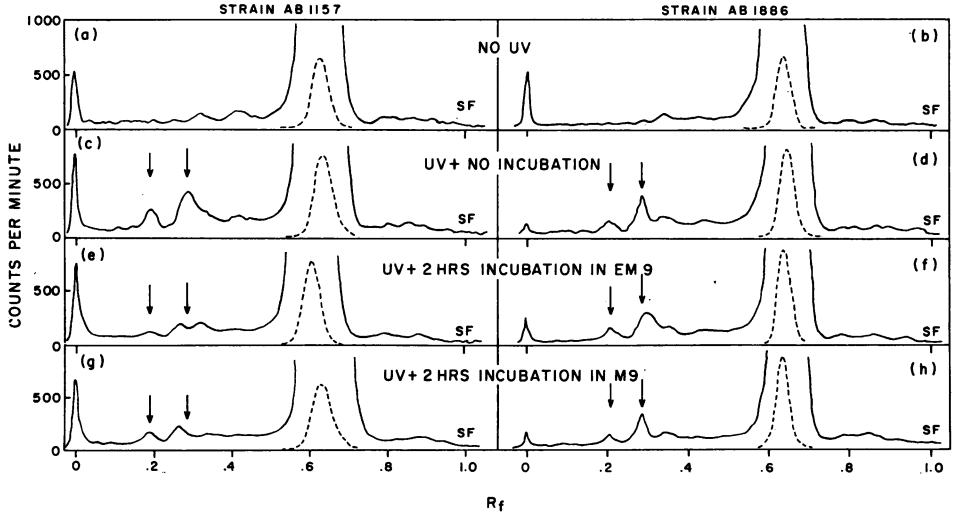

FIG. 1.—Radiochromatograms showing distribution of radioactivity in the acid-insoluble fractions of irradiated and nonirradiated *E. coli* K-12. Cells were labeled with H^3-TdR, irradiated with a dose of 960 ergs/mm^2, incubated, extracted with cold acid, and chromatographed. See *Materials and Methods* for details. Solvent fronts are indicated by SF, and photoproducts by arrows. Activities represented by the dashed lines are 100 times the vertical scale. EM9 is M9 medium containing 0.4 mg/ml casamino acids and 0.1 μg/ml thiamine.

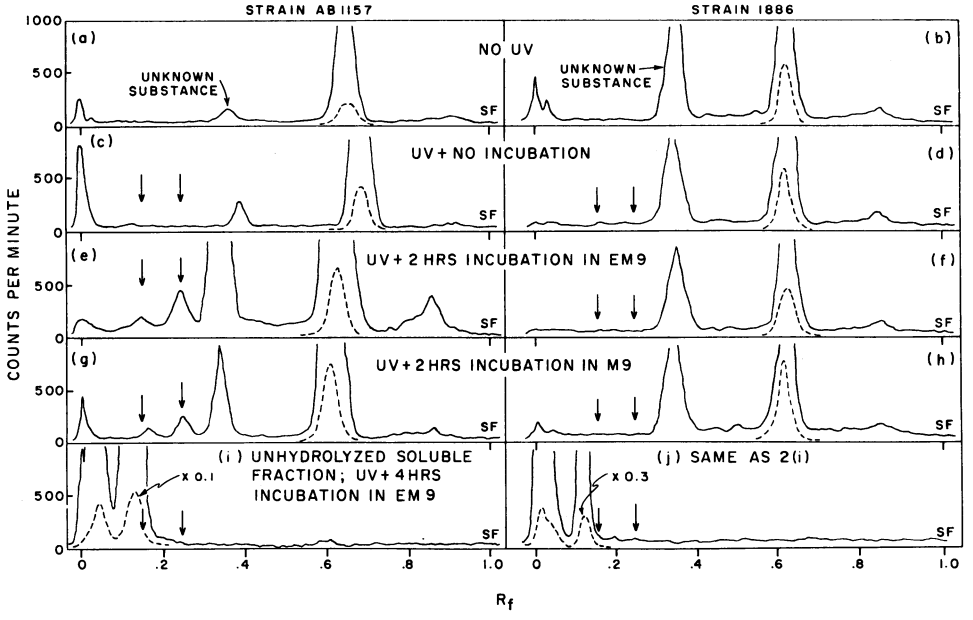

FIG. 2.—(*a–h*) Radiochromatograms showing distribution of radioactivity in acid-soluble fractions hydrolyzed for 90 min in hot acid from the same experiment described in Fig. 1. (*i* and *j*) The same for *unhydrolyzed* soluble fractions of cells irradiated with 900 ergs/mm² and incubated 4 hr in EM9 from another experiment.

as shown in Figure 3*d*. There was a decrease, relative to thymine, of both photoproducts for UV-resistant cells which had been irradiated and incubated for two hr in EM9 or in M9, as seen in Figure 1*e* and 1*g*. The UV-sensitive strain AB1886 was subjected to the same treatment. It is seen in Figure 1 that, although the same photoproducts were produced by irradiation, they did not disappear from the insoluble fraction after postirradiation incubation.

These results suggest that the resistant strain is able to eliminate photoproducts, which include thymine dimer as the principal product (*vide infra*), from irradiated DNA, but that the sensitive strain is unable to do so. Thymine dimers are produced between adjacent thymine molecules in the same DNA strand[16, 17] and their removal could conceivably occur by two mechanisms. They could be split *in situ*, as is possibly the case with irradiated transforming DNA exposed to the photoreactivation enzyme,[10] or they could be excised. Thus, experiments were designed to determine the fate of the thymine dimers. Figure 2 shows radiochromatograms of the acid-soluble fractions which had been hydrolyzed in hot trifluoroacetic acid and which correspond to the insoluble fractions above. Two areas of activity, indicated by arrows in Figures 2*e* and 2*g*, appeared in the UV-resistant strain incubated two hr in either EM9 or in M9 after irradiation. These photoproducts behaved like those in the corresponding insoluble fractions as seen in Figure 3*e* and 3*f*. In another experiment the acid-soluble fractions were chromatographed *without* hydrolysis in hot acid. The results, given in Figure 2*i* and 2*j* for the irradiated, incubated samples of both strains, showed no dimer. These two results indicate that thymine dimers are excised, but that the excision must occur by cleavage of the phosphodiester backbone.

TABLE 1

THE DISTRIBUTION OF RADIOACTIVITY INCORPORATED AS H^3-TdR IN THE ACID FRACTIONS OF UV-IRRADIATED *E. coli* K-12

		H^3 Activity (cpm)						
		Acid-insoluble Fraction				Acid-soluble Fraction		
		1	2			3	4	
Strain	Treatment	Photo-products	Thymine	Ratio 1:2	Change	Photo-products	Thymine	Ratio 3:4
AB1157 *uvr*$^+$	No UV	<100	247,000	<0.04%		<40	6,700	<0.6%
	960 ergs/mm^2-no incubation	1,250	304,000	0.40%	0%	<40	12,000	<0.3%
	960 ergs/mm^2 incubation in EM9*	357	295,000	0.12%	−71%	940	44,500	2.1%
	960 ergs/mm^2 incubation in M9	760	277,000	0.27%	−33%	580	23,500	2.5%
AB1886 *uvr*$^-$	No UV	<100	200,000	<0.05%		<40	6,250	<0.6%
	960 ergs/mm^2-no incubation	805	260,000	0.31%	0%	<40	6,250	<0.6%
	960 ergs/mm^2 incubation in EM9*	890	267,000	0.33%	+6%	<40	4,900	<0.8%
	960 ergs/mm^2 incubation in M9	715	234,000	0.31%	0%	<40	6,450	<0.6%

* EM9: M9 containing 0.4 mg/ml casamino acids and 0.1 μg/ml thiamine.

Table 1 gives the numerical results of the former experiment. The photoproducts/thymine ratio in the AB1157 strain decreased by approximately 70 per cent after two-hr incubation in EM9, and by 30 per cent after incubation in M9. The amount of photoproducts that appeared in the soluble fractions was approximately equal to that which disappeared from the insoluble fractions. The photoproducts/-thymine ratio of 0.3 per cent remained unchanged in the UV-sensitive AB1886 strain, and no detectable thymine dimer appeared in the acid-soluble fractions. These data also show that about 10 per cent of the total thymine was released into the soluble fraction of AB1157 after postirradiation incubation compared with 1–2 per cent in the sensitive strain.

The appearance of two photoproducts after irradiation of DNA labeled with H^3-TdR is of interest. The ratio of the principal photoproduct to the minor one was 2.5 ± 0.26 as determined from 3 independent experiments. When both photoproducts were eluted together and rechromatographed, they appeared in the same relative positions, as seen in Figure 3*c* and 3*e*. The R_f value of 0.28 ± 0.01 for the main photoproduct corresponds to that for reference thymine dimer. When the rechromatographed photoproducts were eluted together, irradiated in water, and chromatographed, only one substance appeared in the position of thymine, as shown in Figure 3*f*. In several other experiments each photoproduct was tested separately for reversion to thymine after irradiation. Typical results are given in Figure 4 showing that each of the resulting products chromatographed like thymine. The above results suggest that the principal photoproduct in the irradiated DNA was thymine dimer. The photoproduct appearing in smaller amounts is as yet unidentified, and may or may not be a stereoisomer of thymine dimer.

Discussion.—When UV-irradiated cells of strain *E. coli* K-12 AB1157 were incubated for two hr at 37° in a minimal medium with or without casamino acids, there

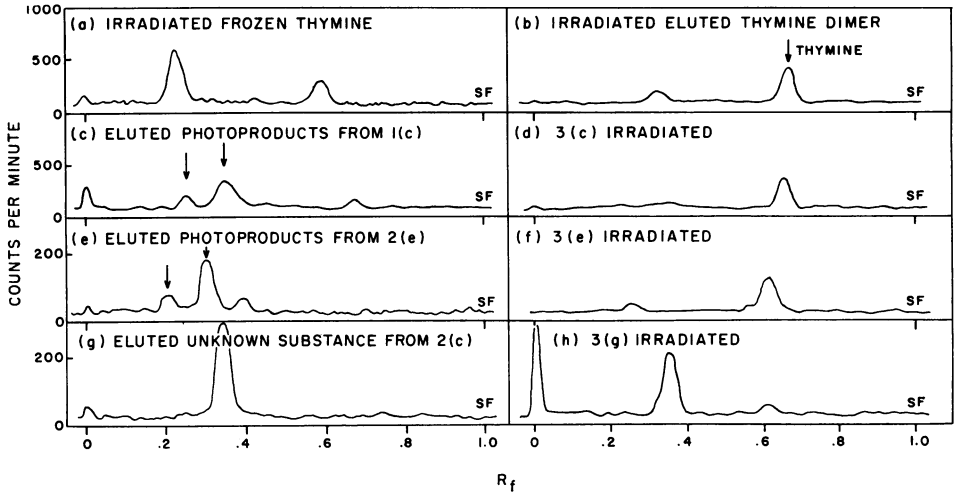

FIG. 3.—UV irradiation of photoproducts. (*a*) Frozen aqueous solution of C^{14}-thymine irradiated with 10^5 ergs/mm^2. The dimer, which chromatographed nearest the origin was eluted in water, UV-irradiated, and rechromatographed to obtain (*b*). (*c–h*) Radioactive photoproducts originating in the fractions indicated of strain AB1157 were eluted from the chromatograms and rechromatographed to give (*c*), (*e*), and (*g*). The active regions in these chromatograms were again eluted, UV-irradiated, and rechromatographed to give (*d*), (*f*), and (*h*).

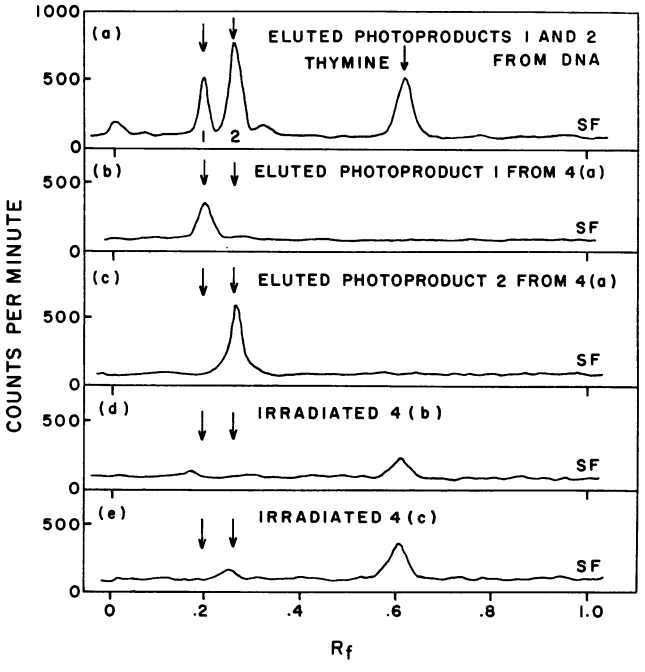

FIG. 4.—Separation and UV irradiation of photoproducts from bacterial DNA.

was a loss of thymine dimers from the incubated acid-insoluble fractions. Moreover, as thymine dimers disappeared from the acid-insoluble fractions, they appeared in the acid-soluble fractions with a photoproducts/thymine ratio 10–20 times greater than in the insoluble fractions. This was not the case in the UV-sensitive strain,

AB1886, derived from AB1157 and apparently differing only in the *uvr*A locus. In this strain, the photoproducts/thymine ratio remained unchanged in the acid-insoluble fractions, and no detectable dimers appeared in the acid-soluble fractions. We interpret these observations as evidence of a dark reactivation process, presumably enzymic, which is capable of excising dimers, some of which may be lethal,[16] from DNA. Thymine dimers, as such, are not removed from DNA, since free dimers are not among the products detected in unhydrolyzed acid-soluble fractions. This indicates that the N-glycosidic bonds are probably not cleaved, but rather that the phosphodiester backbone of the DNA strand containing the dimer is broken. Bollum and Setlow[18] have shown that the phosphodiester bond within a thymine dimer is resistant to nucleases. Therefore, dimers must be extracted as $\widehat{TpT}$, $p\widehat{TpT}$, $\widehat{TpT}p$, $p\widehat{TpT}p$, or as part of a larger fragment. The appearance of about 10 per cent of the total thymine in the acid-soluble fractions after irradiation and incubation of the resistant cells is in contrast to the 1–2% in the sensitive strain. This difference may in part reflect the action of enzymes responsible for dimer excision.

A strand of DNA containing a region of deleted nucleotides might be expected to undergo one of the following possible reactions. (1) Nucleotides complementary to the opposite strand might be inserted into the deleted region with two thymidine molecules replacing the dimer. In this case that section of the DNA strand would function normally. (2) The deleted region may fail to rejoin, or it might perhaps close to form a deletion mutation. (3) The single polynucleotide chain within a deleted region may serve as a favorable site for the initiation of UV-induced genetic recombination with other strands of DNA.[19] This could take place by a breakage and rejoining mechanism, possibly related to that observed with λ phage.[20]

The present results suggest that reactivation of UV-irradiated DNA can occur through the following events. (1) Thymine dimers or other photoproducts are excised from a single polynucleotide chain. (2) Nucleotides are inserted into the excised region of the single DNA strand by complementary pairing with the intact opposite strand. (3) The broken phosphodiester backbone is rejoined. This mechanism for the repair of DNA makes use of the base sequence of the complementary strand of DNA, which may account for the failure of UV-irradiated φX174 phage to be reactivated.[21] The cause of UV sensitivity resulting from a mutation in the *uvr*A-6 locus in strain AB1886 is apparently the failure of a nuclease to excise thymine dimers. A mutation resulting in the impairment of any of the above three functions could conceivably lead to a UV-sensitive mutant. We are presently examining other sensitive mutants, the sensitivity of which is genetically controlled by loci which map in different regions of the chromosome from the *uvr*A locus.

Post X-irradiation degradation of DNA[22] might be related to the enzymatic removal of other radiation products besides thymine dimers from DNA which is then reconstructed in the same way. We are impressed by the potential of this method for the deletion of defects in DNA and the reconstruction of the required base sequence from information on the complementary strand, and by the possibility that it might be employed for the preservation of the DNA of higher organisms.

Summary.—The fate of thymine dimers in DNA during incubation after UV light irradiation was studied in two strains of *E. coli* K-12. One was a multiply auxotrophic strain, AB1157, and the other was a UV-sensitive mutant, AB1886, derived

from it. Strain AB1886 is unable to reactivate UV-irradiated T1 phage and is known to have a mutation at the *uvr*A locus. Cells were labeled in their DNA by growth with H^3-thymidine, exposed to UV light, incubated in enriched minimal medium, and extracted with cold trichloroacetic acid. The acid precipitate and soluble fractions were hydrolyzed in hot acid, and the products were separated by paper chromatography. Thymine dimers were identified in the acid-insoluble fractions from both strains before incubation. During incubation thymine dimers were released into the acid-soluble fraction in the parental strain, AB1157, but not in the UV-sensitive strain AB1886. It is concluded that thymine dimers are excised from the DNA during the reactivation process in the uvr^+ strain and that the sensitive uvr^- strain cannot do this. These findings suggest that the enzymatic removal of injured bases, including thymine dimers, and the reconstruction of the DNA from information on the complementary strand may be an important biological mechanism for the preservation of DNA.

We gratefully acknowledge many stimulating discussions with Drs. R. Setlow, J. Setlow, and E. Canellakis during the course of this work. This work was supported by USPHS grants.

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