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BIOLOGICALLY IMPORTANT DAMAGE TO DNA BY PHOTOPRODUCTS OTHER THAN CYCLOBUTANE-TYPE THYMINE DIMERS *

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Many different kinds of photoproducts are produced in DNA by ultraviolet light (for recent reviews see [1–7]), but the largest amount of published information concerns the chemical nature and biological importance of the cyclobutane-type thymine dimer. The sheer volume of these results has tended to imply that other types of photochemical lesions in DNA are not of biological significance. Quite to the contrary, however, although the thymine dimer is of major importance under certain experimental conditions, this is not true for all situations. The relative importance of the thymine dimer is different for different strains of bacteria and can even change for a given strain under different growth and irradiation conditions. More specifically, the absolute biological importance of the thymine dimer (or of any photoproduct for that matter) depends upon (1) whether or not it is formed under a particular set of experimental conditions and (2) if formed, whether or not the particular system under study is capable of repairing the lesion.

The same data that are used to demonstrate that thymine dimers are of biological importance can be used to suggest the importance of other types of lesions in DNA. The Setlows [8,9]) have demonstrated in a beautiful set of experiments that cyclobutane-type thymine dimers play a major role in the inactivation of irradiated transforming DNA *in vitro*. After low doses of UV, as much as 90% of the damage could be attributed to thymine dimers [10]) or more generally to pyrimidine dimers of the cyclobutane-type since it is now recognized that cytosine forms dimers with itself as well as with thymine (for a recent

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review see [6]). After higher doses of UV only about 50% of the damage could be attributed to cyclobutane-type dimers [8]) suggesting that other types of lesions may also be of importance in the UV inactivation of transforming DNA *in vitro*.

Bacterial cells that have been irradiated with UV show an increased survival if they are additionally irradiated with visible light [11]). This process is known as photoreactivation (for recent reviews see [12] and [13]), and the enzyme responsible for this phenomenon has been shown to be specific for the repair of cyclobutane-type pyrimidine dimers [9,12]). If this enzyme has the same specificity *in vivo* then the ability of cells to be photoreactivated argues for the biological importance of pyrimidine dimers. It should be emphasized, however, that photoreactivation does not cause the complete reversal of all UV damage to a cell. One explanation for this observation is that there are other photochemical lesions produced in DNA besides the cyclobutane-type pyrimidine dimers.

Certain strains of *E. coli* are very sensitive to killing by UV while others are very resistant. The observation has been made by Setlow and Carrier [14]) and by Boyce and Howard-Flanders [15]) that the resistant strains have the ability to cut out thymine dimers from their DNA and by Pettijohn and Hanawalt [16]) that they undergo repair replication of their DNA whereas the sensitive strains are unable to perform this "cut and patch" type of repair. While pyrimidine dimers may be of major biological importance to an organism that is not capable of repairing them, they are probably of little significance to those organisms that have efficient repair mechanisms [17]). Other types of photochemical lesions must therefore lead to the death of these resistant organisms.

This would appear to be particularly true in the case of the extremely radiation-resistant organism *Micrococcus radiodurans*. While the action spectrum for the killing of *E. coli* shows a maximum at about 2600 Å, the action spectrum for the killing of *Micrococcus radiodurans* has maxima both at 2600 Å and at 2800 Å [18]) (fig. 1). Classically a peak at 2800 Å in an action spectrum has indicated protein involvement while that at 2600 Å has indicated the involvement of DNA. It has been suggested by Setlow and Boling [18]) that the resistance of *Micrococcus radiodurans* to UV is due to its ability to repair thymine dimers, but that what ultimately kills this organism is some sort of damage to DNA and to protein. One such type of damage may be the cross-

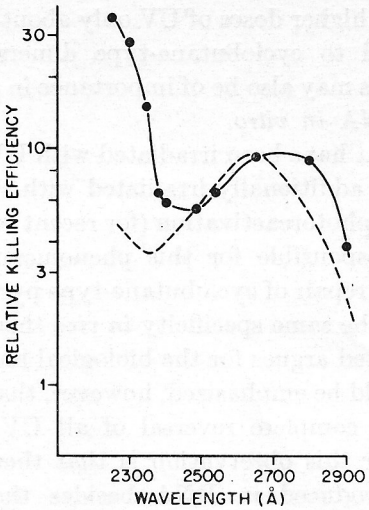

Fig. 1. Action spectra for the killing of *Escherichia coli* H/r 30R and *Micrococcus radiodurans*. Data for *E. coli* given by the dotted line while that for *M. radiodurans* by the solid line. Figure adapted from [18]).

linking of DNA and protein as observed in irradiated *E. coli* by Smith[19]) and by Alexander and Moroson [20]).

When transforming DNA or *E. coli* are irradiated in solution, cyclobutane-type pyrimidine dimers appear to be the photochemical lesion of major but *not of exclusive* biological importance; however there is a growing list of publications indicating that when DNA or *E. coli* are irradiated under different conditions pyrimidine dimers are of little or no importance. For example, they cannot be of biological importance under conditions where they are not formed.

We [21]) have confirmed the results of Riklis [22]) that the formation of thymine dimers is greatly depressed if DNA is irradiated dry. Since bacteriophage T1 cannot be photoreactivated if irradiated dry [22,23]), it implies that pyrimidine dimers are not formed in phage DNA under these conditions. Bacteriophage irradiated while wet is more sensitive to killing by UV than is dry phage and this difference in sensitivity may be due to the formation of pyrimidine dimers in the wet but not the dry phage. However, since the photobiology of dry phage does not seem to involve pyrimidine dimers, other types of lesions must therefore explain the lethal effects of UV under these conditions.

Donnellan and Setlow [24,25]) and Smith and Yoshikawa [21]) have demonstrated that very little of the cyclobutane-type thymine dimer is produced in irradiated bacterial spores but a new photoproduct of thymine is produced in high yield. At high doses of UV, the yield of dimer saturates at about 2% but the new spore photoproduct saturates at about 28% of the thymine present [21]). Bacterial spores are somewhat more resistant to killing by UV than are vegetative cells and this difference in sensitivity may be explained in part by the relative yield of thymine dimers; however, since cyclobutane-type thymine dimers appear to play only a minor role in the photobiology of spores, other types of lesions must therefore explain the lethal effects of UV on spores.

Mammalian cells do not appear to be able to repair thymine dimers by photoreactivation [26,27]) or by excision [26–28]), and Trosko and Kasschau [29]) have detected thymine dimers in UV-irradiated mammalian cells that were still viable. Although an unrepaired thymine dimer seems to be lethal to a bacterial cell this would not appear to be the case for mammalian cells. Thymine dimers therefore do not appear to be of unique importance to mammalian cells.

We [30]) have confirmed the observation by Ashwood-Smith *et al.*[31]) that when bacteria are irradiated while frozen they show an enhanced sensitivity to killing by UV (fig. 2). Furthermore, the relative sensitivity to killing is a function of the temperature at which the cells re irradiated [30,31]). In going from room temperature to −79 °C, there is a decreased production of cyclobutane-type thymine dimers for a given dose of UV but a new photoproduct of thymine is produced that seems to be identical with the new photoproduct of thymine isolated from irradiated spores [30]) (fig. 3). As mentioned previously, the increased resistance of spores to UV as compared to vegetative cells seems to be correlated in part with a decreased production of thymine dimers. In the freezing experiments, however, we have an inverse correlation between UV killing and the production of thymine dimers [30]). This inverse correlation suggests that cyclobutane-type dimers do not play as significant a role in the events leading to the death of irradiated frozen cells as they do at room temperature. We have some preliminary information suggesting that the enhanced UV sensitivity of frozen cells may be explained in part by an enhanced cross-linking of DNA and protein by UV [30]).

The cross-linking of DNA and protein *in vivo* by UV has been

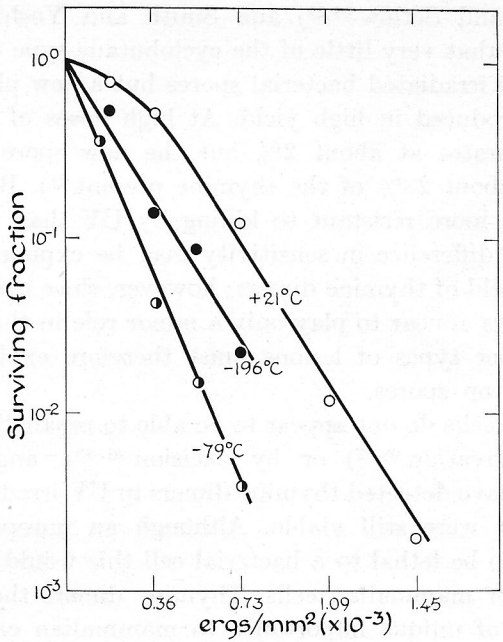

Fig. 2. Survival curves for *Escherichia coli* B/r, T⁻ as a function of the temperature during UV irradiation (2537 Å).

demonstrated by Smith *et al.*[30,32]) to be of biological importance under certain conditions. This phenomenon was originally observe as a dose-dependent decrease in the amount of DNA that could be isolated free of protein subsequent to UV irradiation [19,20]). We find this response to be analytically many times more sensitive than thymine dimer formation *in vivo*. Thus, at the 99% killing dose for *E. coli* B/r (1800 ergs/mm²) only 0.1% of the thymine was converted to the thymine dimer, yet the same dose rendered 11% of the DNA unextractable [19]). The amount of the DNA that was lost from the soluble phase due to irradiation could be quantitatively accounted for in the precipitate containing the denatured proteins. Treatment of this material with trypsin, however, yielded free DNA. These data suggested that the DNA was cross-linked to protein. Further proof came from experiments showing that DNA and protein could be cross-linked *in vitro* [5,33]) (fig. 4).

The precise chemical mechanism by which DNA and protein are cross-linked is not known, however, our recent isolation of a dimer of

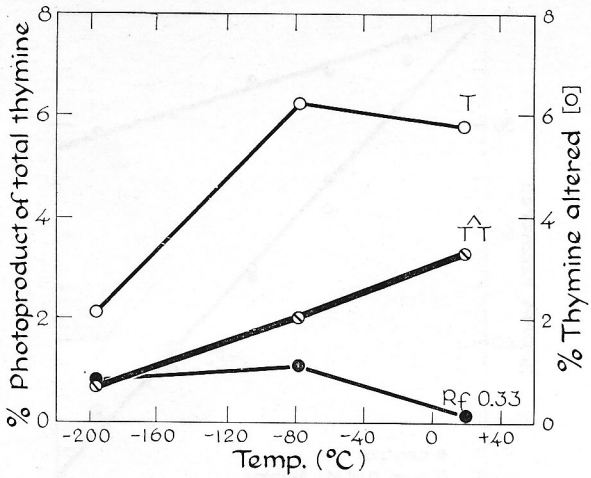

Fig. 3. Thymine photoproduct formation in *Escherichia coli* B/r, T⁻ as a function of the temperature during UV irradiation (2537 Å). The cells labeled with thymine-2-C ^{14} were irradiated with 2×10^4 ergs/mm^2, hydrolyzed in trifluoroacetic acid and chromatographed in n-butanol/acetic acid/water (80/12/30). T strands for thymine and $\widehat{TT}$ for the cyclobutane-type thymine dimer (Rf 0.26). Other radioactive areas were observed at Rf 0.0, 0.08, 0.13, 0.19 and 0.41. The yield of these products generally decreased with temperature.

uracil and cysteine (5-S-cysteine, 6-hydrouracil) (fig. 5) after UV irradiation *in vitro* of a solution of uracil and cysteine may serve as a possible model for the cross-linking phenomenon [34]). Cysteine-S-35 adds photochemically to poly-uridylic acid, poly-cytidylic acid and to DNA [35]) and we are currently trying to determine if the point of attachment of the cysteine to DNA is exclusively through the cytosine residues.

The presence of 0.01 M alanine does not affect the UV-induced cross-linking of *E. coli* DNA and bovine serum albumin *in vitro*. However, the presence of 0.01 M cysteine greatly inhibits this reaction (fig. 4). Presumably the cysteine is competing with the SH (or OH) residues on the protein for attachment to the cytosine residues in the DNA [36]). Gelatin contains no SH groups and cross-links with DNA very poorly. The fact that gelatin cross-links at all, and that the presence of 0.01 M cysteine further reduces the cross-linking ability of gelatin suggests that there may indeed be other mechanisms for the cross-linking of DNA and protein besides those involving SH groups [36]).

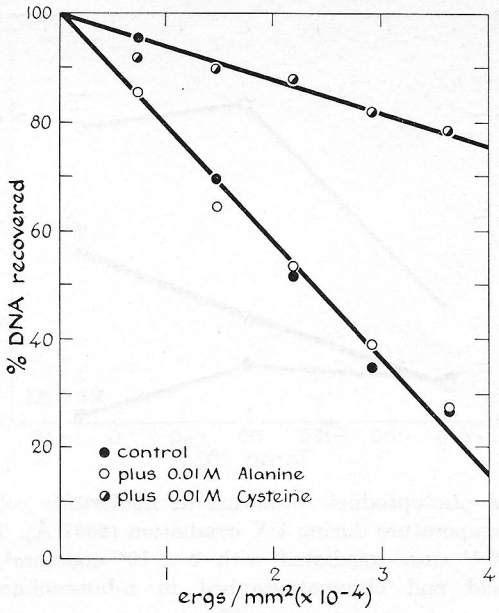

Fig. 4. The photochemical cross-linking of DNA and protein *in vitro*. 4.2 ml of water containing 10 mg bovine serum albumin, 0.03 mg DNA-thymine-2-C^{14} (8.85×10^4 cpm/OD_{260} unit) and where indicated, 0.042 mM of either alanine or cysteine. The solutions were irradiated (2537 Å) for various times and aliquots were processed for the recovery of DNA [19]). It has been previously shown that there is no loss of DNA when irradiated in the absence of protein [5,33]).

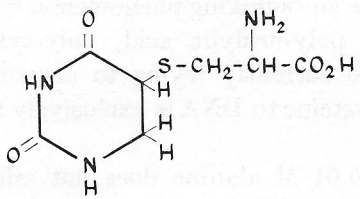

Fig. 5. 5-S-cysteine, 6-hydrouracil.

Cells that have been grown on bromouracil instead of thymine are more sensitive to killing by UV [37]) and they are also about five times more sensitive to UV-induced DNA-protein cross-linking than are normal cells [5]). The findings by Haug [38]) that cysteine adds to irradiated TpBU and by Rupp and Prusoff [39]) that cystamine adds to irradiated iodouracil to form 5-S-cysteamine uracil and to irradiated DNA containing bromouracil [40]) may explain the enhanced cross-linking observed in cells whose DNA contains bromouracil, and in

turn, may help explain the increased sensitivity of these cells to killing by UV.

We have observed that conditions that photoreactivate colony formation in UV-irradiated bacteria have no effect upon the amount of DNA cross-linked to protein [5]). Holding the cells after irradiation in non-nutritive solution also had no effect upon the amount of DNA cross-linked to protein when corrected for its appropriate unirradiated control [36]). These results would tend to suggest that DNA-protein cross-links are not repaired; however, if very large pieces of DNA containing the cross-linked protein were excised this would probably be assayed as showing no repair by our present assay technique.

The biological importance of the cross-linking of DNA and protein was first indicated by our studies in which changes in DNA-protein cross-linking were correlated with changes in the sensitivity of *E. coli* 15 TAU to killing by UV during growth in selected nutritionally deficient media [32]). The most dramatic situation studied was thymine starvation although the other conditions studied led to similar conclusions. During thymine starvation there was a cyclic change in the percentage of cells surviving after a given dose of UV (fig. 6). The changes in sensitivity to killing were accompanied by similar directional and time-sequential changes in the amount of DNA that became cross-linked to protein by a constant dose of UV (fig. 6). The near equivalence in the timing of these changes in the sensitivity to killing and in the cross-linking of DNA and protein by UV suggest that under these conditions the cross-linking phenomenon must play a significant role in the loss of viability of these irradiated cells. Since it has been shown that RNA synthesis [41]) and more particularly messenger RNA synthesis [42]) is necessary before thymine starvation becomes lethal, the major protein cross-linked to DNA under conditions of thymine starvation may be the DNA-directed RNA-polymerase. However, in normal log phase cells, that portion of the genome that is involved in replication (i.e., pulse-labeled section of the DNA) is the most sensitive to cross-linking to protein [33]).

I mentioned previously that it has been observed that bacteria irradiated in the frozen state are more sensitive to UV irradiation [30,31]) (fig. 3), even though fewer thymine dimers of the cyclobutane-type are formed under these conditions [30]) (fig. 4). This inverse correlation would suggest that some lesion other than thymine dimers must account for the greater sensitivity of *E. coli* to UV radiation in the

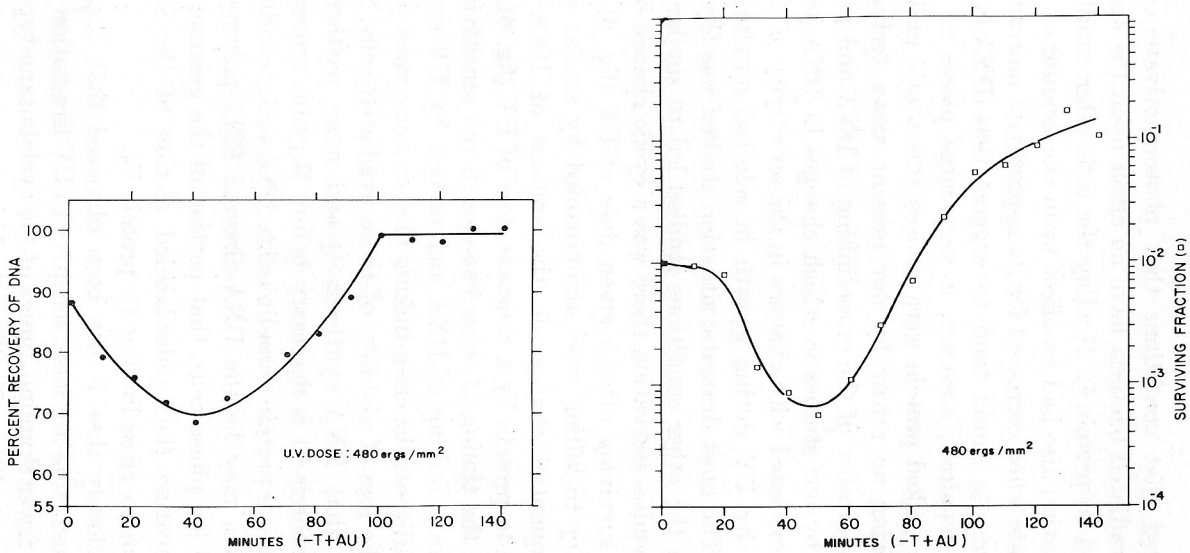

Fig. 6. The susceptibility of *Escherichia coli* 15 TAU to killing and to the cross-linking of their DNA and protein by ultraviolet light (2537 Å) at various times of growth in the absence of thymine. A logarithmic culture of *E. coli* 15 TAU was suddenly switched to a medium devoid of thymine. At various times thereafter, two aliquots were withdrawn from the culture. One of these aliquots was irradiated and then both aliquots were assayed for viable cells and the amount of DNA that could be extracted free of protein. Figure adapted from Smith *et al.* [32]).

frozen state. We find that the photochemical event that seems to correlate with the increased sensitivity to killing is the increase in the cross-linking of DNA and protein [30]). There appears to be both a change in rate and yield in the cross-linking of DNA and protein with freezing (fig. 7). When the per cent cross-linking at a given dose

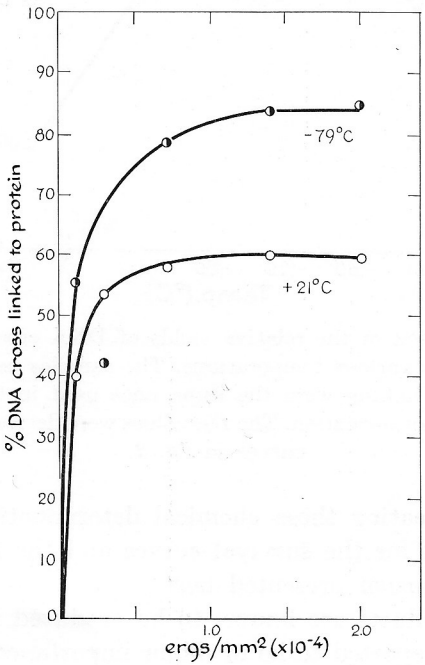

Fig. 7. Cross-linking of DNA and protein in *Escherichia coli* B/r, T⁻ as a function of the UV dose (2537 Å) at different temperatures. The experimental technique for the extraction of DNA is given in [19]).

of UV is compared with the sensitivity to killing, expressed as the reciprocal of the D_{37} dose, there is a good correlation between the results at $-79°$ and $-196°$ but a poor correlation at room temperature (fig. 8). The poor correlation at room temperature is comforting because we know already that the photoproduct of major biological importance at room temperature is the thymine dimer and not DNA-protein cross-links, but it would appear that in the frozen state the enhanced killing can be correlated with DNA-protein cross-linking. These results should be considered preliminary since they compare chemistry at one dose of UV and viability at another; however, we

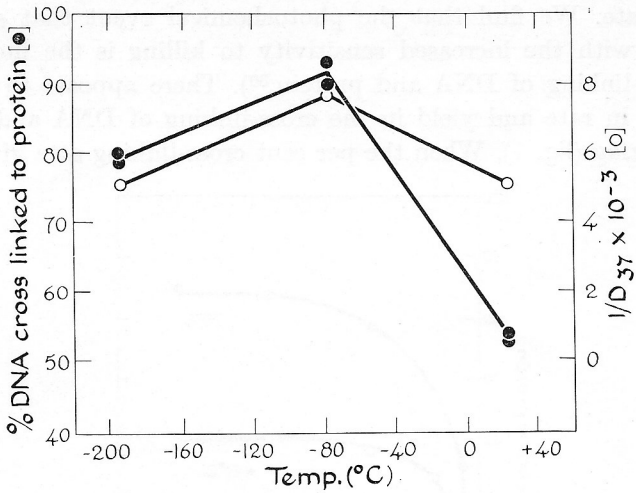

Fig. 8. A comparison of the relative yields of DNA cross-linked to protein and the D_{37} dose at various temperatures. The samples used for determining the extent of cross-linking were the same ones used in fig. 3 to determine thymine photoproduct formation. The D_{37} values were derived from the survival curves in fig. 2.

are currently repeating these chemical determinations at the same doses of UV used for the survival curves and the results appear to confirm the conclusion presented here.

Other photoproducts are known to be produced in DNA [1–7]), but these have been reported to be of minor importance. The evaluation of the relative importance of these photoproducts was done, however, under conditions where pyrimidine dimers are now known to be of major biological importance. It would therefore seem of value to redetermine the relative role of these photoproducts under conditions where the pyrimidine dimers appear to be only of minor biological significance (i.e., dry and frozen cells, spores, etc.). The photoproducts I am referring to are pyrimidine hydrates, DNA-DNA cross-linking, chain breakage and new photoproducts of thymine and cytosine which have been identified chromatographically but as yet are not chemically defined.

The possible role of pyrimidine hydrates in causing mutations has been demonstrated *in vitro* in Grossman's laboratory. When polycytidylic acid was irradiated with UV, its coding properties in an RNA-polymerase system were altered [43]). The irradiated polymer lost

its ability to code for the incorporation of guanylic acid but then coded for the incorporation of adenylic acid. Heating the irradiated poly-cytidylic acid, under conditions known to repair pyrimidine hydrates, prevented incorporation of adenylic acid and for this reason it was suggested that the code change might be the result of the formation of cytosine hydrates [43]).

Present evidence indicates that hydrates of cytosine are not formed in irradiated double-stranded DNA but are formed in irradiated single-stranded DNA [44]). Therefore, the formation of cytosine hydrates in single-stranded regions of DNA may well be of significance in the production of mutations which may or may not be lethal. Growth and irradiation conditions that favor the greater single-stranded character of DNA should favor the production of hydrates. One would predict that mutation frequencies might be greater in cells irradiated in log phase than in those irradiated just before division where presumably their DNA is in a more quiescent double-stranded form. Since hydrates are known to be repaired by heat, a systematic re-evaluation of heat reactivation of cells and viruses might now be worthwhile (for a review of heat reactivation see [4]) p. 342).

Marmur *et al.*[45]) found that the efficiency of DNA chain breakage brought about by UV irradiation of DNA *in vitro* was not greatly affected by the base composition of the DNA. The dose of UV required to reduce the molecular weight of DNA to 50% was about 100 times that required to reduce the transforming activity of the same DNA to 50% [45]). At the dose of UV required to kill 99% of a population of phage T7, no chain breaks were detected [46]). Each of these determinations were done under conditions where thymine dimers appear to be of major biological importance. It might be of value to again look for the relative importance of chain breaks under conditions where thymine dimers appear to play a reduced role in lethality. Also, the more sensitive technique of sucrose density gradient centrifugation for evaluating molecular weight changes might yield new information.

DNA-DNA cross-links leading to gel formation have not been observed in UV-irradiated wet cells [20]) but have been observed in DNA irradiated while dry [47,48]), and in UV-irradiated salmon sperm heads [20]) where the DNA is known to be very tightly packed. It is interesting therefore that although pyrimidine dimers are suspected to be involved in the formation of DNA-DNA cross-links [48]) the conditions that favor their formation do not favor the formation of

cyclobutane-type thymine dimers. DNA-DNA cross-links may well achieve a position of greater biological importance when cells and viruses or DNA are irradiated dry.

Another type of DNA-DNA cross-linking causes the two strands of a single molecule of DNA to be cross-linked so that they can no longer be made to completely separate when treated with heat or formamide [49]). For a given dose of UV the extent of cross-linking increased proportionally with the adenine-thymine content of the DNA samples, suggesting that some type of dimer of thymine might be responsible for the cross-linking [49]); however, the chemical nature of these cross-links is still unknown. From structural considerations it is highly unlikely that the usual form of the cyclobutane-type thymine dimer would be responsible for these interstrand cross-links. DNA in which almost all of the thymine was replaced by bromouracil was about five times more sensitive to interstrand cross-linking by UV than was normal DNA [50]). No interchain cross-links were detected by Freifelder and Davison [46]) in phage T7 irradiated to a survival of 1%. However, since it has been shown that partial denaturation favors interstrand cross-linking [51]), growth or irradiation conditions that might induce partial single strandedness in the DNA might bring this lesion to a position of greater biological importance.

Many pyrimidine photoproducts other than the hydrates or the cyclobutane-type dimers are produced both *in vitro* and *in vivo*. The structures of most of these new photoproducts are unknown and their identity is based upon their chromatographic properties ([21,24]), and further references in [6])). The relative production of certain of these products does vary with different experimental conditions, but their biological importance must still be determined.

This talk should not be construed as an argument against the importance of thymine dimers but only an argument that they are not of major significance in all situations. I have cited several conditions where pyrimidine dimers have been shown to play a major role and I have cited several examples where they appear to be only of minor importance.

Another photochemical lesion that appears to assume major importance under certain conditions is the cross-linking of DNA and protein. Proof of its importance has relied upon a correlation between the enhanced cross-linking of DNA and protein and the enhanced killing of cells under certain growth and irradiation conditions.

Although it is recognized that correlations do not necessarily prove causal relationships, it nevertheless appears that the only way to prove that a photochemical event within a cell is of biological importance is to gather correlative data. A perfect correlation between survival curves and rate of formation of a given photoproduct will probably never be achieved since UV irradiation causes the formation of many types of photoproducts within a cell, all of which make their contribution to lethality. Under certain growth or irradiation conditions these photoproducts may each in turn be elevated to a position of major importance. The relative role of any photochemical lesion must therefore be determined under each new set of experimental conditions.

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