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THE BIOLOGICAL IMPORTANCE OF U.V.-INDUCED DNA-PROTEIN CROSS-LINKING *IN VIVO* AND ITS PROBABLE CHEMICAL MECHANISM*

KENDRIC C. SMITH
Department of Radiology, Stanford University School of Medicine, Palo Alto, California 94304, U.S.A.

Abstract—The increased sensitivity of *Escherichia coli* to killing by u.v. while frozen and the changes in sensitivity as a function of temperature during irradiation have been correlated with the increased production of DNA-protein cross-links and have been shown not to be related to thymine dimer production. These results provide yet another example where thymine dimers make little contribution to the u.v. inactivation of a biological system (but does not detract from the biological importance of thymine dimers in other systems). We have reported that uracil and cysteine can combine to form 5-S-cysteine, 6-hydrouracil when a solution of these two compounds is irradiated with u.v. (2357 Å). This led us to offer this compound as a model for the mechanism by which DNA and protein are cross-linked *in vivo*. We have now extended our studies to the kinetics of the photochemical addition of ^{35}S-cysteine to various polynucleotides and to DNA. Selected rate constants for cysteine addition (μM cysteine/μM nucleotide involved /erg/mm^2 $\times$ 10^8) are as follows:

poly rU 21·4
poly rC 8·1
poly dC 2·6 = (poly dC : dG) = (poly dT : dA)
poly dT 5·4

The rate constants for native and heat-denaturated DNA can be accounted for by the summation of the rate constants for the appropriate polynucleotides. The addition of cysteine to RNA shows a biphasic response probably related to the amount of single-stranded regions present. Preliminary results indicate that tyrosine and serine also add photochemically to DNA.

IT IS becoming increasingly apparent that the relative yield of the different types of photoproducts that are produced in DNA can vary depending upon the type of organism used, the growth state of the organism, the irradiation conditions, and more particularly, upon the physical state and the environment of the DNA during irradiation (for review see Smith[1]). Although the thymine dimer was the first lesion shown to be of biological importance, there is an ever-increasing mass of data to document that there are many situations in which thymine dimers play little or no role in u.v. inactivation [1]. The implication is, of course, that in these situations photochemical lesions other than the dimers are responsible for the inactivations seen. Of the situations in which the dimer is known to be of little or no biological importance, such as spores, dry bacteriophage, and frozen bacteria, the lesion responsible for inactivations is known only for bacteria. The increased sensitivity of *Escherichia coli* to killing by u.v. while frozen and the changes in sensitivity as a function of temperature during irradiation have been correlated with the increased production of DNA-protein cross-links and have been shown not to be related to thymine dimer production[2].

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Our variant of *E. coli* B/r showed differences in survival after u.v. irradiation as a function of the temperature at which the cells were irradiated (Fig. 1), in agreement with the results of Ashwood-Smith *et al.*[3]. When the temperature was reduced from +21°C to −79°C, an increase in sensitivity to u.v. radiation was shown both by a change in extrapolation number and a change in the slope of the survival curves. At −196°C the cells were not as sensitive as at −79°C, but were more sensitive than at +21°C[2].

A larger percentage of DNA was cross-linked to protein by a given dose of u.v. radiation when the cells were irradiated at −79° or at −196°C as compared to +21°C (Fig. 2). There is clearly a correlation in rank between the several cross-linking curves in Fig. 2 and the survival curves in Fig. 1. This correlation has recently been confirmed by Bridges *et al.*[4].

In contrast, the rate of formation of thymine dimers decreased when the temperature of the cells during irradiation was varied from +21° to −79°C and to −196°C (Fig. 3). These curves show no correlation with the survival curves in Fig. 1[2].

Concomitant with this decrease in yield of thymine dimers in irradiated frozen cells, a decrease in the production of photoreactivable damage also occurred. This was seen when the cells at +21° and −79°C were either exposed to the same dose of u.v. radiation or were killed to approximately the same survival value (Fig. 4). Since current evidence suggests that the photoreactivating enzyme is specific for the repair of cyclobutane-type pyrimidine dimers[7], the reduced amount of photoreactivation is consistent with the observed decrease in production of thymine dimers under these conditions[2].

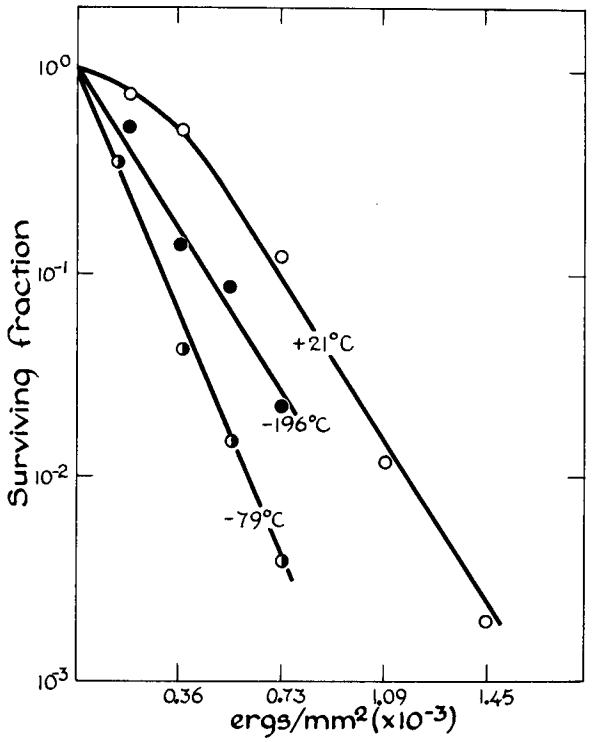

Fig. 1. Survival of *E. coli* B/r, T⁻ as a function of u.v. dose (2537 Å) at different temperatures (from Smith and O'Leary[2]).

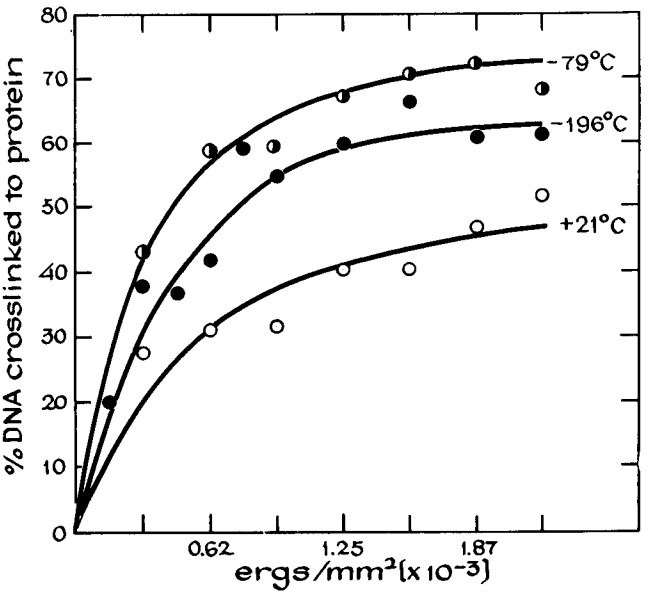

Fig. 2. Cross-linking of DNA and protein in *E. coli* B/r, T^- as a function of u.v. dose (2537 Å) at different temperatures. The values plotted are the average values for five experiments at +21.C and two each at − 79° and − 196°C (from Smith and O'Leary[2]).

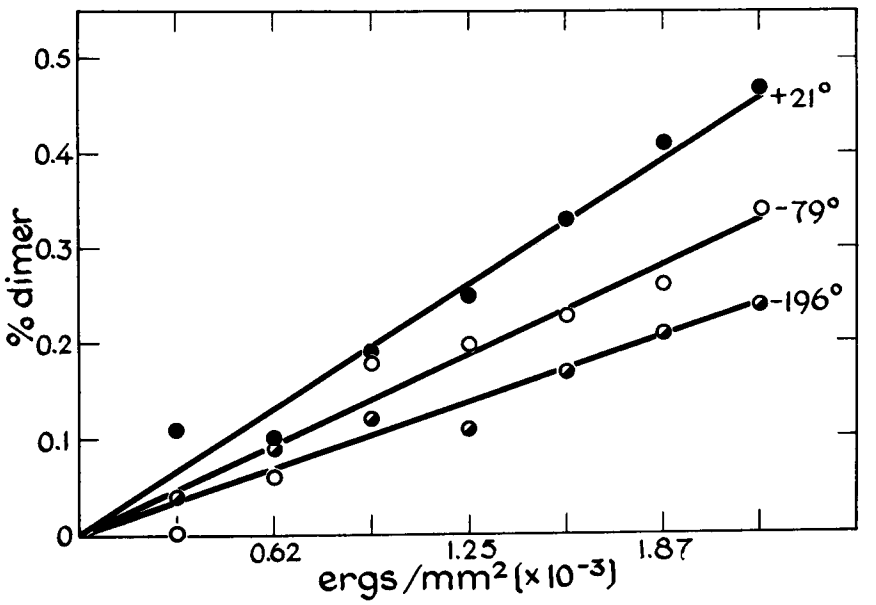

Fig. 3. Formation of cyclobutane-type thymine dimers in *E. coli* B/r, T^- as a function of u.v. dose (2537 Å) at different temperatures. Cells labeled with thymine-2-^{14}C were irradiated, hydrolyzed in trifluoroacetic acid, and chromatographed[5, 6]. The cells used were from certain of the experiments described in Fig. 2. These results are the average of two experiments at +21°C and one each at the other temperatures (from Smith and O'Leary [2]).

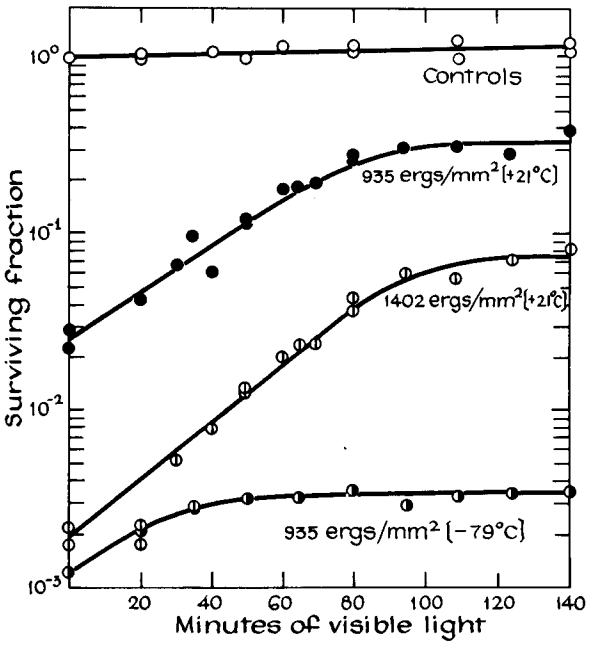

Fig. 4. Photoreactivability of *E. coli* B/r, T^- after u.v. irradiation (2537 Å) at +21° and −79°C. Stationary cultures were suspended (5×10^8 cell/ml) in 0·1 *M* phosphate buffer at pH 6·8. The suspension was frozen at −79°C for 30 min and irradiated, or thawed and irradiated at +21°C. Samples on a shaker table were then exposed to visible light at +21°C for the times indicated, and viability was determined on plates of nutrient agar. The visible-light source was a pair of 15 W Westinghouse Daylight fluorescent bulbs 9 cm above the bottom of the Petri dish. The top half of the glass Petri dish was inverted and filled with 15 ml of water (from Smith and O'Leary[2]).

There is no correlation between the production of thymine dimers and the increased killing of *E. coli* by irradiation at −79° and −196°C. This suggests that cyclobutane-type thymine dimers do not play as significant a role in the events leading to the death of irradiated frozen cells as they appear to play at room temperature[8]. These results provide further evidence that the relative biological importance of a given photoproduct can change markedly, depending upon growth or irradiation conditions[1].

The photochemical event that does correlate with viability under the present conditions is the cross-linking of DNA with protein. Freezing produces both a change in the rate of formation and in the yield of DNA cross-linked to protein. Freezing may alter the configuration or the proximity of the protein and DNA within the cells so that the probability of forming DNA-protein cross-links by irradiation is greatly enhanced, thus leading to the greater lethality observed under these conditions.

Although the biological importance of the u.v.-induced cross-linking of DNA and protein has been demonstrated[2, 9], the nature of the chemical linkage involved is still unknown. The technical complexities of using a bacterial system caused us to search for a suitable *in vitro* system with which to study the chemical nature of the linkage group(s). We had shown that purified *E. coli* DNA would cross-link readily with bovine serum albumin[10, 11], and that a mixed photoproduct of cysteine and uracil (5-S-cysteine, 6-hydrouracil) (Fig. 5) could be formed when uracil and cysteine

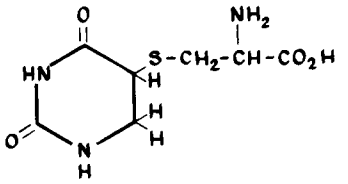

Fig. 5. 5-S-Cysteine, 6-hydrouracil. This product is formed when a solution of uracil and cysteine is irradiated at 2537 Å[12].

were irradiated together in solution[12]. This mixed photoproduct of cysteine and uracil was offered as a possible model for the cross-linking of DNA and protein, but since uracil is not present in DNA, the question arose as to whether cytosine and/or thymine would undergo this type of addition with cysteine. We have therefore investigated the photochemical reactivity of ^{35}S-cysteine with various polymers containing uridylic acid, cytidylic acid, and thymidylic acid as well as RNA and DNA[13].

After irradiation the polynucleotide was precipitated with alcohol and redissolved in saline. An aliquot was then submitted to gel filtration on Bio–Gel P–2 to separate the polynucleotide from any residual unreacted ^{35}S-cysteine. The amount of polynucleotide in the peak was determined by microphosphate analysis, and the amount of ^{35}S-cysteine photochemically combined with the polynucleotide was determined by analysis of the radioactivity present. The micromoles of cysteine combining per micromole of phosphorous per dose of u.v. could then be calculated. The results for these experiments are given in Table 1.

Table 1. Rate constants for the photochemical addition of ^{35}S-cysteine to polynucleotides*

Polynucleotide	K†	
	Experimental	Calculated
Poly rU	21·8 (13·3)‖	
Poly rU : rA	— 0·7‖ (U only)	
Poly rA	0·6	
Poly rC	8·1 (0·6)‖	
RNA (yeast)‡	21·8 (20% C + 27% U)	
	4·8	
Poly dC	2·6	
Poly dC : dG	2·6 (C only)	
Poly dT	5·4	
Poly dT : dA (heated)§	4·2 (T only)	
Poly dT : dA	2·6 (T only)	
Poly dAT : dAT	1·1 (T only)	
DNA (calf thymus)	2·6 (21%C + 29%T)	2·6 (dC : dG + dA : dT)
DNA (heated)§	4·2	4·2 (dC + dT)

*From Smith and Meun[13].

†$K = \dfrac{\mu\text{moles cysteine}/\mu\text{moles PO}_4\text{ involved}}{\text{ergs/mm}^2} \times 10^8$ (at pH 5).

‡RNA shows a biphasic uptake of cysteine.

§15′ at 100°C in 0·075 *M* NaCl. Quick cooled.

‖At pH 6·5.

Of all of the polymers tested, single-stranded poly rU showed the greatest photochemical reactivity with cysteine, whereas the two-stranded polymer poly rU:rA took up almost no cysteine. It is therefore of interest that Pearson and Johns[14] found that the rate of hydration in poly rU:rA was suppressed by a factor of 10 relative to poly rU and the rate of dimer formation by a factor of 5.

That the uptake by poly rU is decreased significantly by raising the pH from 5 to 6·5 suggests that the photochemical reaction may involve an ionic species of either uracil or cysteine. The pK values for cysteine are 1·96, 8·18, and 10·28[15], and that for uracil is approximately 10·5[16]. One would therefore not expect a significant change in the ionization of either cysteine or uracil in going from pH 5 to 6·5. However, the involvement of the ionic form of uracil (probably in the excited state) in the photohydration reaction has been reported by Burr and Park[17], and there are some similarities between the photohydration reaction and the addition of a molecule of cysteine to the 5–6 double bond of uracil[12].

The rate of uptake of cysteine at pH 5 by poly rC was less than half of that observed under similar conditions for poly rU (Table 1). At pH 5, poly rC should be mainly (but not completely) in a two-stranded form[18]. When poly rC was assayed at pH 6·5, at which it should be single-stranded, there was almost no photochemical uptake of cysteine. The protonated form of cytosine therefore seems to be required both for the formation of double-stranded molecules of poly rC and for the efficient uptake of cysteine.

At pH 5 there is a greater uptake of cysteine by poly rC than by poly dC (Table 1). This may possibly be due to the lesser degree of double-strandedness in poly rC at pH 5.

The dC residues in poly dC:dG should have the same configuration as in double-stranded poly dC. Consistent with this supposition is the fact that the rate of uptake of cysteine was the same both for two-stranded poly dC and for poly dC:dG (Table 1).

The uptake of cysteine by poly dT was completely unexpected, inasmuch as thymine is not reported to undergo hydration-type reactions[19]. When we try to explain the results for the uptake of cysteine by two-stranded polymers containing dT, we are faced with several apparent inconsistencies. In agreement with the results for the uridylic acid polymers but in contrast to those for the cytidylic acid polymers, the rate of uptake of cysteine by double-stranded poly dA:dT was only about half that for single-stranded poly dT. A heated and quick-cooled sample of poly dA:dT took up more cysteine than an unheated sample. However, our sample of poly dAT:dAT took up almost no cysteine. These results could be taken to suggest that poly dAT:dAT was more tightly hydrogen-bonded than poly dA:dT and would predict a higher T_m for poly dAT:dAT. Unfortunately, the T_m for poly dAT:dAT has been reported to be 7·5°C lower than for poly dA:dT[20]. The tightness of the helical structure would appear then not to be the only factor involved in determining the rate of the uptake of cysteine by polymers containing dT.

When cysteine reacts photochemically with uracil, its sulfur attaches to carbon 5 of uracil and a hydrogen adds to carbon 6 to give 5-S-cysteine-6-hydrouracil[12]. Pershan *et al.*[21] have shown that when DNA is irradiated with u.v., a thymine radical is formed by the addition of hydrogen to carbon 6, leaving an unpaired electron on carbon 5. Recently, Yamane *et al.*[22] identified dihydrothymine as a u.v. irradiation product of thymine in DNA and observed that its production appeared to follow the

thymine radical yield. One may speculate that the addition of cysteine to thymine in DNA might follow a similar reaction scheme to yield 5-S-cysteine-6-hydrothymine and would therefore be analogous in structure to the cysteine-uracil photoproduct. We have recently isolated a mixed photoproduct of thymine and cysteine and are in the process of determining its chemical structure.

Denatured DNA took up cysteine at almost twice the rate of native DNA. Furthermore, the rate constants for heat-denatured and native DNA were accurately predicted (Table 1) from the rate constants for the appropriate single- and double-stranded deoxy polymers. It has been pointed out by Riley *et al.*[20] that "the environment of the AT pair in DNA appears to be intermediate between that of the homopolymer pair and that of the strictly alternating helix." This conclusion would not appear to hold for the photochemical reactivity of cysteine with the different AT pairs. The AT pair in DNA would appear to have the same photochemical reactivity towards cysteine as does the AT pair of poly dA:dT, but differs markedly from the AT pair in poly dAT:dAT[13].

In contrast to all the other polymers studied, RNA showed a biphasic uptake of cysteine versus dose of u.v. Approximately 3 per cent of the pyrimidines took up cysteine at about the rate exhibited by poly rU, whereas the second slope of the curve represented a rate only slightly more than half that of poly rC[13].

In order to obtain some preliminary information relevant to the nature of the linkage between cysteine and the several polymers, we have studied the heat stability of the various cysteine-polymer photoproducts. The peak tubes containing the photoproduct were pooled from several analytical experiments, alcohol-precipitated, and redissolved in a small amount of water. One sample served as the unheated control and was run through the gel filtration column, the peak was recovered, and the micromoles of cysteine taken up per micromole of phosphorous were determined. Other samples were heated at 65°C (the temperature currently used during the extraction of DNA from u.v.-irradiated bacterial cells[2]) for various times before being put on the gel filtration column. The ^{35}S-cysteine-DNA photoproduct showed a linear loss of radioactivity with time of heating up to 30 min and then showed no further change even after 90 min of heating. A maximum of 36 per cent of the cysteine was removed from the DNA by heating at 65°C. Similar data for the other polymers heated for 60 min at 65°C are presented in Table 2.

Table 2. Heat stability (60 min at 65°C) of amino acid photoadducts to polynucleotides*

	Per cent stable photoadduct†	
	Cysteine	Tyrosine
DNA	64 (70)‡	34
Poly dC	51	
Poly dT	84	
Poly rU	99	

*Adapted from Smith and Meun[13].
†Produced at pH 5; stability tested in saline.
‡Calculated from the results for poly dC and poly dT.

The ^{35}S-cysteine–poly rU photoproduct appears to be completely stable under the conditions used. Furthermore, when the ^{35}S-cysteine–poly rU photoproduct was partially acid-hydrolyzed and the hydrolysate chromatographed, a material with the same R_f of authentic 5-S-cysteine, 6-hydrouracil was isolated and was shown to chromatograph identically with authentic 5-S-cysteine, 6-hydrouracil in three additional solvents. We conclude therefore that the cysteine adds to position 5 of poly rU as it does when combining with monomeric uracil[13].

Only about 50 per cent of the ^{35}S-cysteine–poly dC adduct was heat stable, suggesting that there may be two types of addition reactions occurring. 85 per cent of the ^{35}S-cysteine–poly dT adduct was heat stable, suggesting that the major photoproduct was through a heat-stable linkage and only about 15 per cent of the adduct was through a heat labile linkage.

Under conditions similar to those used to test the photochemical reactivity of cysteine with DNA, we have tried preliminary experiments with other amino acids. These data are given in Table 3. Tyrosine appears more reactive than cysteine, whereas serine is less reactive. Threonine and methionine show marginal reactivity. The tyrosine adduct to DNA is less stable to heat than the cysteine adduct (Table 2).

Table 3. Rate constants for the photochemical addition of various amino acids to DNA*

	Cysteine	Tyrosine†	Serine†	Threomine†	Methionine†
K‡	2·6	5·5	0·7	0·2	0·2

*Adapted from Smith and Meun[13].
†Determined at pH 5 for only one dose point ($4{\cdot}8 \times 10^5$ ergs/mm^2).
‡Rate constant; see Table 1 for definition.

Now that we have an indication that certain amino acid adducts to DNA have a measurable lability to heat, we can profitably discuss the possible reasons why we have been essentially unsuccessful in isolating the cross-linked DNA-protein complex in purified form from u.v.-irradiated bacteria. We have tried to purify this complex by solubilizing it in various detergents at 60–65°C and by gentle shearing so that this complex would not sediment to the bottom but would appear somewhere in the middle of a sucrose gradient. However, after these treatments we find that the protein is no longer bound to the DNA. Furthermore, an experiment in which ^{35}S-labeled cells were digested with pronase in the hope of finding increased amounts of ^{35}S-cysteine attached to the DNA with increasing doses of u.v. failed to give the expected results (K. C. Smith, unpublished observations). This failure may be due to the heat lability of the cysteine linkage, or it may mean that cysteine is not the amino acid primarily concerned with *in vivo* DNA-protein cross-linking. The finding that tyrosine is even more reactive than cysteine suggests that the pronase experiment should be repeated using ^{14}C-tyrosine to label the cells.

The lability of certain amino acid adducts to the nucleic acids may also explain the results of Goddard *et al.*[23] who found for u.v.-irradiated tobacco mosaic virus that one molecule of protein was cross-linked per molecule of RNA per lethal hit. The cross-linked protein-RNA complex withstood sodium lauryl sulfate at 50°C for 5 min

but was split by subsequent treatment with phenol, 5 M quanidine hydrochloride, or 66 per cent acetic acid.

Two reactions of the pyrimidines that have been described by other authors may have some importance in the photochemical cross-linking of DNA and protein. Alcantara and Wang[24] have observed the formation of 5-formyluracil when thymine is irradiated in the solution with u.v. If this reaction occurs in irradiated DNA, the formyl group could then react with a protein amino group to give a covalent bond between DNA and protein[25].

Janion and Shugar[26] have observed that dihydrocytosine will react with glycine such that the amino group of the dihydrocytosine is replaced by the amino group of the glycine, resulting in a covalent link between dihydrocytosine and glycine. Since both the photohydrate and cyclobutane-type dimer of cytosine are analogs of dihydrocytosine, one may predict that the addition of protein amino groups to these cytosine photoproducts might serve as another mechanism by which DNA and protein are cross-linked by u.v.

Both of these reactions are secondary to the primary photochemical event produced in the DNA. It is therefore of interest to recall that *in vitro* cross-linking was obtained between *E. coli* DNA and bovine serum albumin even when the protein and/or the DNA were irradiated separately and then mixed[10]. However, holding irradiated bacteria (*E. coli* B/r or 15 TAU) in water for various times (up to 8 hr) after irradiation (360 and 720 ergs/mm^2 at 2537 Å) did not change the yield of DNA cross-linked to protein (when corrected for their unirradiated controls) (K. C. Smith, 1964, unpublished observations).

It would appear that the phenomenon of u.v.-induced DNA-protein cross-linking can now take its place alongside the thymine dimer as a photoproduct of biological importance. The chemistry of this reaction *in vivo* is still to be elucidated, but there are now several examples of chemical reactions that can occur between pyrimidines and different acids. Much exciting photochemistry should result from experiments to elucidate the mechanisms by which amino acids combine with the nucleic acids.

Most of the photochemical work on DNA to date has been on pure systems, but DNA does not exist in a pure system within a cell. The DNA is constantly bathed by cellular constituents such as proteins, RNA, and metabolites. The finding that DNA can combine photochemically with amino acids suggests that more attention should be given to these addition reactions in trying to explain the biological effects of u.v., especially when the effects cannot be adequately explained by known photoproducts produced in pure DNA.

RESUMEN

La pronunciada sensibilidad del *Escherichia coli* al ser irradiado por luz u.v. cuando está congelado y los cambios en la sensibilidad con la temperatura durante la radiación han sido relacionados con el aumento en la producción de uniones ADN-proteina y se ha demostrado no tener relación con la formación de dímeros de la timina. Los resultados aquí presentados proveen otro ejemplo para ilustrar que los dímeros de la timina practicamente no contribyen a la inactivación con luz u.v. de un sistema biológico (pero no niega la importancia biológica de los dímeros de la timina en otros sistemas). Hemos reportado que el uracilo y la cisteina pueden combinarse para formar 5-S-cisteina. 6-hidrouracilo cuando una solución de estos dos componentes es irradiada con luz u.v. (2537 Å). Esto nos conduce a presentar a este compuesto para explicar el mecanismo por el cual el ADN y la proteina se unen in vivo. En la actualidad hemos extendido nuestros estudios a la cinética de la adición fotoquímica de ^{35}S-cisteina a varios polinucleótidos y al ADN. Las

constantes de velocidad para la adición de cisteina en (μM de cisteina/μM de nucleotido/erg/mm$^2 \times 10^8$) son las siguientes:

poli rU 21·4
poli rC 8·1
poli dC 2·6 = [poli dC : dC] = [poli dT : dA]
poli dT 5·4

Las constantes de velocidad para el ADN nativo y desnaturalizado por el calor pueden obtenerse sumando las constantes de velocidad de los polinucleótidos correspondientes. Algunos resultados preliminares indican que la tirosina y la serina tambien se adicionan fotoquímicamente al ADN.

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