# CONCERNING THE MECHANISM OF FORMATION OF UV-INDUCED THYMINE PHOTOPRODUCTS IN DNA*

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A cyclobutane-type homodimer of thymine (T=T)[1] formed from adjacent thymines in the same polynucleotide strand[2] and the analogous dimers of adjacent pyrimidines in general[3–5] are currently considered to be responsible for most of the adverse biological effects produced by ultraviolet (UV) irradiation. Although this concept has greatly expedited conceptual formulation and actual experimentation in UV photobiology, the chemical evidence supporting these identifications in DNA[6] is somewhat thin, consisting more of intelligent extrapolations from experiments with model systems than a rigorous chemical study of DNA photoproducts themselves. The T=T identification has been based mainly on the similarities between a product obtained from acid hydrolysates of UV-irradiated DNA and the identified photodimer formed from massive irradiation of thymine in frozen solutions (e.g., $R_f$ values on paper chromatograms, and the reversion to material having the $R_f$ of thymine after UV irradiation in solution) with the support of less direct evidence.[7–9] This is also true for a second thymine-containing product,[10] surmised to be a heterodimer U=T arising from deamination of photoinduced C=T.[4, 5]

From studies of the mechanism of photochemical reactions of pyrimidines in frozen solutions and dried films,[11] it seems clear that the stereochemical requirement for the cyclobutane-type dimerization is quite rigid. The relative positions of adjacent pyrimidines in one strand of a Watson-Crick double helix do not appear to favor the *direct* formation of a cyclobutyl-type structure in DNA. From these considerations, it might then be expected that one or more sterically favored intermediates are necessary if such dimers are to form.

Identification of photoproducts derived from thymine and 5-bromouracil suggests the formation of dithymine peroxide (TOOT) and a thymine-coupled product (T—T) as possible, but still undetected, intermediates.[12] Intrastrand formation of either TOOT or T—T would not only be sterically accommodated in the normal Watson-Crick structure but could also lead to the formation of T=T and/or other products. An investigation of these possibilities was therefore undertaken; reported below are the first results of such a study, strongly suggesting the existence of intermediates in the formation of thymine photoproducts.

*Materials and Methods.—Labeled bacterial DNA for irradiation in vivo:* For labeling of cellular DNA, 100 ml of M9 medium[13] containing 2 $\mu$g/ml of radioactive thymine was inoculated with *Escherichia coli* 15 T$^-$. After 15–18 hr of rotary shaking at 37°, the cells were harvested by centrifugation, washed twice with 0.1 *M* potassium phosphate buffer (pH 7), resuspended in 80–150 ml of the same buffer, and shaken for 1 hr at 37°. Portions of the suspension were irradiated as described below. (Samples taken after 7 hr growth gave the same results.)

*Labeled DNA for irradiation in vitro:* Bacteria were harvested from late log-phase cultures grown as described above, washed and resuspended to *ca.* $5 \times 10^{10}$ cells/ml in 0.15 *M* NaCl-0.015 *M* sodium citrate, containing 26% (w/v) sucrose. Procedures used for lysis and purification of DNA were those described elsewhere.[14] The purified DNA (750 $\mu$g/ml; specific activity

$2.7 \times 10^4$ dpm/$\mu$g DNA) was stored in saline-citrate buffer, and diluted 100-fold prior to irradiation.

*Labeling of cellular DNA:* For single-label experiments, thymine-2-$C^{14}$ (25.2 mc/mM, CalBiochem Corp.; 40.2 mc/mM, Nuclear-Chicago Corp.) had a specific activity of 0.1 $\mu$c/$\mu$g. For double-label experiments, thymine-5-methyl-$H^3$ (13.3 c/mM, New England Nuclear Corp.) was added to the desired $H^3/C^{14}$ activity ratio (varying between 1:1 and 10:1), with the specific activity of thymine-5-methyl-$H^3$ at 0.1 to 2.0 $\mu$c/$\mu$g.

*Experiments involving solutions of labeled thymine:* For single-label experiments, aqueous solutions of thymine-2-$C^{14}$ (3 mM, 0.4 mg/ml) had a specific activity of $6 \times 10^{-4}$ $\mu$c/$\mu$g. For double labeling, the solution also contained thymine-5-methyl-$H^3$ at specific activities of $6 \times 10^{-4}$ to $4.5 \times 10^{-2}$ $\mu$c/$\mu$g.

*Irradiation conditions:* Samples (10-ml) in 9-cm Petri dishes were irradiated at room temperature with an unfiltered 15-w G.E. germicidal lamp at a dose rate of approximately 20 ergs/mm$^2$/sec, as measured and routinely monitored by a Latarjet dosimeter.[15] Cell suspensions were handled under yellow light after UV irradiation.

*Irradiation of DNA solutions at various pH values:* Purified, thymine-2-$C^{14}$-labeled DNA was denatured (by heating at 100°C for 5 min followed by quenching in ice), and the concentration adjusted to 7.5 $\mu$g/ml in 0.15 *M* NaCl and buffered with 0.007 *M* sodium citrate, 0.001 *M* glycine, and 0.001 *M* potassium phosphate, pH 7. For the acid range, an aliquot was brought to pH 1.9 with 6 *N* HCl, and 20-ml aliquots were titrated with 10 *N* NaOH to the desired pH, then irradiated with a dose of $1.0 \times 10^4$ ergs/mm$^2$. Following the irradiation, 2.5 ml of 1 *M* potassium phosphate buffer, chosen to produce a final pH of 7, was added to each sample. An analogous procedure was used for the alkaline range, the solution being titrated to pH 12.0 with 10 *N* NaOH, and back-titrated with 6 *N* HCl to the desired pH. Controls were: unirradiated and irradiated denatured DNA kept at neutral pH; unirradiated DNA previously titrated to the extreme acid or alkaline pH and neutralized with and without a subsequent irradiation; and neutral-irradiated DNA titrated to pH 1.9 or 12.0, held for 40 min at room temperature before neutralizing. Samples were dialyzed vs. distilled water, then dried, hydrolyzed, and chromatographed.

*Postirradiation treatment:* (1) *Cell suspension:* Irradiated cell samples were washed twice with 10 ml of cold 5% TCA and twice with 10 ml of ethanol:ether (3:1). The dried samples were hydrolyzed in 0.5–1.5 ml of trifluoroacetic acid (TFA) for 80–120 min at 170–180°C in sealed tubes,[16] then spotted directly for chromatography on Whatman no. 1 paper. Descending chromatography was carried out at room temperature using different eluents (cf. Fig. 1). (2) *DNA solutions:* Irradiated samples of DNA were dialyzed vs. 30 vol of distilled water at 5°C, changed three times, reduced to dryness in a flash evaporator, and taken up in trifluoroacetic acid for hydrolysis and subsequent chromatography in the same manner as described for cell suspensions. (3) *Frozen-irradiated thymine solutions:* The thawed solution (10 ml for each run) was evaporated to dryness, taken up in 2.0 ml of 0.1 *N* HCl, and divided into ten 0.20-ml portions for chromatography.

*Radioactivity measurements:* The dried chromatograms were analyzed for distribution of radioactivity with a Vanguard Autoscanner 880. Each peak area of radioactivity was cut out and eluted with water. Eluates of 3–8 ml were collected for each sample, evaporated to dryness, and the entire sample was taken up in 10 ml of scintillation solution (ref. 18, without ethylene glycol); the vials were counted in a Nuclear-Chicago model 723 scintillation spectrometer. For thymine peaks, only $^1/_{100}$ of the total sample was used for scintillation counting. The counting efficiency was determined by internal standardization using standard toluene samples. For single labeling ($C^{14}$), the counting efficiency was 73%; under double-labeled conditions, the average efficiencies in these experiments were for $C^{14}$: channel 1 = 15.5%, channel 3 = 50.6%; and for $H^3$: channel 1 = 9.6%, channel 3 = 0.42%.

*Results.—Thymine-derived compounds from DNA irradiated in vivo and in vitro:* Two radioactive peaks (in addition to that of thymine) have been revealed in hydrolysates of DNA irradiated *in vivo* by paper chromatography.[4, 7, 10] Separation of these products in several different solvent systems is shown in Figure 1. The peak migrating with $R_f = 0.20$ in solvent system A is designated in this paper[18] as $P_1$; the

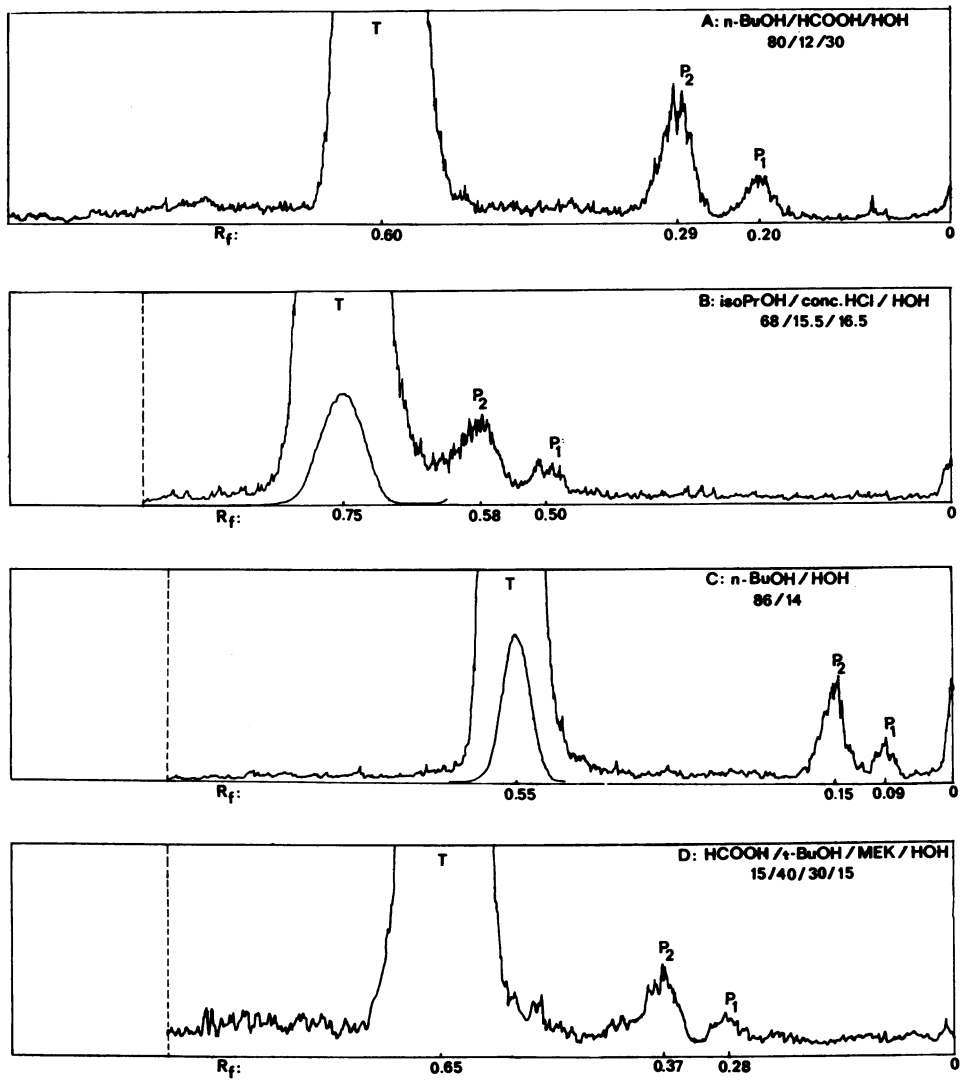


FIG. 1.—Radiochromatograms showing distribution of radioactivity in the acid hydrolysates of *E. coli* 15 $T^-$ irradiated *in vivo*. Cells were labeled with thymine-2-$C^{14}$ and irradiated with a dose of $1.9 \times 10^4$ ergs/mm$^2$. Solvent systems, and $R_f$ values of $P_1$, $P_2$, and thymine are indicated; the dashed lines represent the solvent fronts. Total activities are the same in all cases, and are represented by the inset curves under the major band of thymine (i.e., total activity = (100/3) $\times$ ordinate value).

other with $R_f = 0.29$, as $P_2$. Good separation of these products is obtained for UV doses (254 m$\mu$) ranging from $2.4 \times 10^3$ ergs/mm$^2$ to beyond $3.9 \times 10^4$ ergs/mm$^2$.

In contrast to the *in vivo* results, the DNA irradiated *in vitro* gives four radioactive peaks. It appears that two are chromatographically identical with the $P_1$ and $P_2$ from irradiated cells.[5] The two additional products are as yet unidentified, and appear not only in irradiated, but also in *unirradiated*, samples, having $R_f$ values of 0.37 and 0.45 in solvent system A and carrying radioactivity of approximately 5

per cent and 8 per cent, respectively, that of thymine.[19] They are distinct from $P_1$ and $P_2$, and occur in about the same amounts for native or heat-denatured, acid- or alkali-treated DNA, from either irradiated or unirradiated samples.

The present paper is concerned with $P_1$ and $P_2$. We have checked that both products revert chromatographically[20] to the $R_f$ of thymine when separately eluted and reirradiated in solution.[5, 10] It is important to note for later discussion that reirradiation of $P_1$ gave no $P_2$ or other product chromatographically different from thymine. Moreover, when the eluted products were resubjected to the same hot-acid conditions under which DNA was initially hydrolyzed, neither product gave new chromatographic peaks of radioactivity. The dose dependence of $P_1$ and $P_2$ formation *in vivo* was essentially that reported by Setlow and Carrier for *in vitro* studies:[5] $P_1$ reaches early saturation with a half-maximum dose of $4.6 \times 10^3$ ergs/mm$^2$, while $P_2$ increases approximately linearly beyond $10^4$ ergs/mm$^2$; but in our experiments the DNA irradiated *in vitro* showed a rate of formation and, in the case of $P_1$, a total amount of product at saturation appreciably greater than the *in vivo* result (half-maximum dose for $P_1$ formation $= 2.9 \times 10^3$ ergs/mm$^2$).

*Involvement of thymine-methyl hydrogens in the formation of $P_1$ and $P_2$:* Based on previous studies,[12] two possible intermediates in $P_1$ and $P_2$ formation might reasonably be postulated (see introduction), each of which involves hydrogen removal from the 5-$CH_3$ group of thymine. In order to test for this removal, cells were doubly labeled with a mixture of thymines carrying tritium at the 5-$CH_3$ group and $C^{14}$ at the 2-carbon position of the ring, respectively. The labeled cells were irradiated and hydrolyzed, and the $H^3/C^{14}$ ratio in the chromatographically separated products was examined by two-channel scintillation counting. The occurrence of a reaction involving the loss of one H on the methyl group would result in a reduction in the $H^3/C^{14}$ ratio for one or both of the (presumably homogeneous) products. On the other hand, direct photodimerization through 5,6-double bonds should not affect the methyl hydrogens, leaving the $H^3/C^{14}$ ratio unchanged in the products.

The results seen in Table 1 show an average reduction of 30 per cent in the $H^3/C^{14}$

TABLE 1

REDUCTION OF THE $H^3/C^{14}$ RATIO IN $P_1$ AND $P_2$ FROM DNA IRRADIATED *in vivo*

| Experiment | Dose (min) | $r$ = (H³ counts)/(C¹⁴ counts) $r_T$ | $r_{P_1}$ | $r_{P_2}$ | Mean value for experiment |
|---|---|---|---|---|---|
| 1 (Four dupl. samples per dose) | 8 | 1.16 | 0.743 | 0.763 | $r_{P_1}/r_T = 0.630 \pm 0.04$ |
| | 16 | 1.15 | 0.760 | 0.718 | |
| | 24 | 1.18 | 0.716 | 0.807 | $r_{P_2}/r_T = 0.637 \pm 0.03$ |
| | 32 | 1.17 | 0.720 | 0.705 | |
| 2 (Two dupl. samples per dose) | 0 | 0.724 | — | — | |
| | 8 | 0.722 | 0.535 | 0.536 | $r_{P_1}/r_T = 0.732 \pm 0.05$ |
| | 16 | 0.707 | 0.530 | 0.489 | $r_{P_2}/r_T = 0.696 \pm 0.05$ |
| | 32 | 0.774 | 0.556 | 0.516 | |
| 3 (Two dupl. samples per dose) | 0 | 8.26 | — | — | |
| | 2 | 8.08 | 5.27 | 4.81 | $r_{P_1}/r_T = 0.709 \pm 0.09$ |
| | 4 | 7.93 | 6.32 | 5.44 | |
| | 8 | 8.49 | 6.31 | 6.53 | |
| | 16 | 8.26 | 5.27 | 6.65 | $r_{P_2}/r_T = 0.747 \pm 0.1$ |
| | 32 | 8.38 | 6.65 | 7.34 | |

The quantity, $r$, refers to the ratio of the calculated disintegrations per min of $H^3$ to that of $C^{14}$ in the separately eluted and counted product fractions. Values of $r_p/r_T$ were averaged, in each experiment, for all values of $P_1$ and $P_2$. In all experiments, analysis of the variances (Bartlett's test), using logarithms of the individual replicate sample ratios, showed no appreciable nonhomogeneity among the ratios. The variances of the individual doses were therefore pooled for the calculation of the average value, $r_p/r_T$. The fiducial limits around each mean $r_p/r_T$ ratio was calculated by the $t$ test (95% confidence level; theoretical value of $r_p/r_T = 0.667$), using the logarithm of these mean values.

ratio for *both* $P_1$ and $P_2$. The same result was obtained with different doses of UV and with different initial $H^3/C^{14}$ ratios in the thymine, and was unchanged when the conditions of hydrolysis were varied (e.g., volume of TFA used (0.5–1.5 ml), duration (80–120 min), and temperature (160–180°C)).

The $H^3/C^{14}$ ratios were also determined following elution and re-exposure to acid hydrolysis conditions, and compared with those obtained initially. We observe no further reduction in the $H^3/C^{14}$ ratio for $P_1$, $P_2$, or thymine as a result of a second hydrolysis, indicating that the loss of tritium does not arise from gradual exchange during hydrolysis but rather during the course of $P_1$ and $P_2$ formation.

These results appear to be consistent with the removal of hydrogen from the 5-$CH_3$ group at an intermediate step in the formation of $P_1$ and $P_2$. On the other hand, it might be argued that the decreased $H^3/C^{14}$ ratio results merely from a primary and/or secondary isotope effect. Obviously, direct dimerization of thymine does not involve cleavage of the C—H bond as a rate-determining step; thus, an isotope effect on the rate of hydrogen removal would not change the essential conclusion that the 5-$CH_3$ group is involved in $P_1$ and $P_2$ formation. If a secondary isotope effect is considered possible, it might be argued that the $H^3/C^{14}$ ratio arises from differential rate of dimerization of thymine carrying —$CH_3$ and —$CH_2T$,[21] respectively, so that they contribute in different degrees to the amount of product formed. We believe this explanation of the result unlikely, however, because isotopic substitution affects essentially only the vibrational and rotational, rather than the electronic energy terms.[22] Tritium substitution must therefore produce molecular energy changes very much smaller (<1 kcal/mole) than the photon excitation "activating" this reaction (113 kcal/mole at 254 m$\mu$), in marked contrast to the situation for thermal reactions; any effect on reaction rates should be correspondingly much smaller.

*Behavior of photoproducts from frozen solution of thymine:* The usual model compound for comparison with thymine-containing products in DNA is the crystalline homodimer recovered from frozen aqueous solutions of thymine after massive irradiation. However, frozen solutions of thymine-2-$C^{14}$ exposed to moderate doses of UV light ($1–2 \times 10^4$ ergs/mm$^2$) give *two* additional peaks of radioactivity, besides thymine, upon paper chromatography.[23] Only the major peak has the chromatographic properties of T=T isolated after massive doses of irradiation. The $R_f$ values of these peaks, designated here as $PT_1$ and $PT_2$, in solvents A, B, and C are the same as those shown for $P_1$ and $P_2$, respectively, in Figure 1. At a dose of $2.0 \times 10^4$ ergs/mm$^2$, the radioactivity in $PT_1$ is about 10 per cent that of $PT_2$.

On reirradiation in aqueous solution *or* upon heating in TFA, $PT_1$ gave *two* products, the $R_f$ values of which agree with those of $PT_2$ and thymine as described earlier by Smith.[23] In contrast, $PT_2$ on reirradiation in aqueous solution formed only one new product, with the $R_f$ of thymine, and its chromatographic behavior was unaffected by acid hydrolysis conditions (Table 2). Thus, although $PT_2$ may be identical with $P_2$, $PT_1$ differs from $P_1$ in spite of its similar chromatographic behavior.

When a mixture of thymine labeled with tritium at the 5-$CH_3$ group and with $C^{14}$ at the 2-carbon position was irradiated in frozen solution and the products were separated chromatographically, an average reduction of 34 per cent of the $H^3/C^{14}$ ratio was observed in $PT_1$, as compared with that of recovered thymine (Table 3). However, *no* such reduction was observed for $PT_2$ (i.e., T=T). This means that

TABLE 2

IRRADIATION AND HYDROLYSIS OF $PT_1$ AND $PT_2$

| Product | $PT_1$ Irradiation | | | $PT_1$ Hydrolysis | | | $PT_2$ Irradiation | | $PT_2$ Hydrolysis |
|---|---|---|---|---|---|---|---|---|---|
| $R_f$ values | 0.16 | 0.27 | 0.60 | 0.17 | 0.27 | 0.60 | 0.27 | 0.60 | 0.27 |
| Radioactivity (%) | 89 | 6.0 | 4.6 | 39 | 21 | 30 | 65 | 35 | 100 |

Irradiation dose = $1.8 \times 10^4$ ergs/mm²; chromatography carried out in solvent A.

TABLE 3

$H^3/C^{14}$ RATIO IN $PT_1$ AND $PT_2$ FROM THYMINE IRRADIATED IN FROZEN SOLUTION

| Expt. | Species | $r = H^3/C^{14}$ | | $r_{P_x}/r_T$ |
|---|---|---|---|---|
| 1 | T | 14.8 | (±1.40) | 1.000 |
| | $PT_1$ | 9.98 | (±0.54) | 0.674 |
| | $PT_2$ | 14.7 | (±0.58) | 0.993 |
| 2 | T | 0.915 | (±0.03) | 1.000 |
| | $PT_1$ | 0.545 | (±0.07) | 0.650 |
| | $PT_2$ | 1.08 | (±0.01) | >1.000 |

The irradiation doses used were: for expt. 1, $1.8 \times 10^4$ ergs/mm²; for expt. 2, $3.6 \times 10^4$ ergs/mm.² The quantity in parentheses refers to the standard error of the mean of four duplicate samples per species.

although $PT_2$ and $P_2$ are similar in a number of ways, they are apparently formed by different mechanisms. If $PT_2$ is indeed identical with $P_2$, it could account for the fact that the quantum yield for dimerization of thymine is much greater in frozen solution than in DNA. At any rate, it would appear that the analogy between thymine dimerization in the solid state and photoproduct formation in the Watson-Crick helix is somewhat less impressive than formerly seemed the case.

*Effect of irradiation pH on $P_1$ and $P_2$ formation:* The suggestion that any thymine homodimer formation in DNA proceeds indirectly can be strengthened by studying the effects of irradiation pH on formation of $P_1$ and $P_2$. Both $P_1$ and $P_2$ are related to photoreactivable damage, since their yields are greatly reduced by enzymatic photoreactivation of the irradiated DNA.[5] (We have confirmed this result, which was expected on the basis of earlier experiments with the chromatographically unresolved pair of products.[24]) The amount of photoreactivable damage is also known to be affected by the pH at the time of irradiation.[25, 26] The results of these experiments (Fig. 2), using previously denatured $C^{14}$-labeled DNA to avoid hysteresis effects from denaturation during titration, show that the two products are also greatly, but differently, affected by the irradiation pH. Control experiments show no lability of either $P_1$ and $P_2$ to a 40-minute treatment of DNA at pH 1.9 or 12.0 following irradiation, and no effect of acid or alkali pretreatment of the DNA on their formation upon subsequent irradiation at neutral pH.

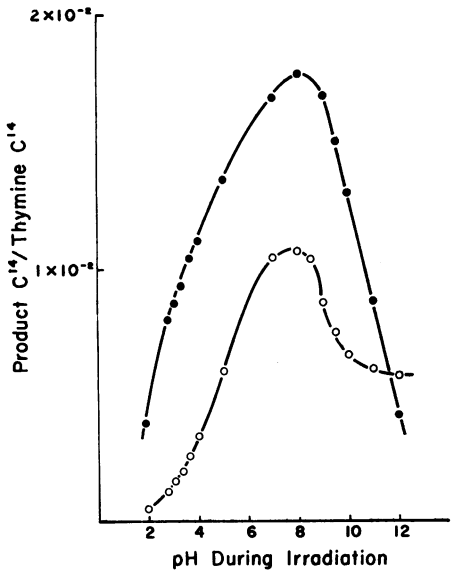


FIG. 2.—The per cent formation of products $P_1$ (—○—○—) and $P_2$ (—●—●—) in irradiated heat-denatured DNA as a function of the pH during irradiation. The thymine-2-$C^{14}$ was exposed to a dose of $1.0 \times 10^4$ ergs/mm²; see text for further details.

The observed decrease of both products on the low-pH side cannot be attributed to pH-dependent changes in the ultraviolet absorbancy of the thymine moieties,

since the maximum absorbancy of the nucleoside or the nucleotide does not change with pH in this region.[27] Indeed this base has no group with an acid $pK_a$ within the pH range used here. This means that if $P_2$ is a homogeneous compound representing T=T, the effect of the irradiation pH on its formation must be exerted via some intermediate reaction step.

*Discussion.*—One is attracted to the concept that cyclobutane-type dimers (T=T, C=T, C=C) formed between adjacent pyrimidines in DNA are responsible for the most significant biological changes induced by UV, because of the range of experimental results they can accommodate.[9] It is nevertheless difficult to reconcile such direct dimerization in a Watson-Crick double helix with the apparently rather rigid steric requirements found for pyrimidine dimerization in the solid state.[11] Associated with this difficulty is an almost total lack of information about the mechanisms involved in dimer, or other pyrimidine photoproduct formation under UV irradiation, and a consequent ignorance about the actual range of product possibilities.

The present study—showing a reduction in $H^3/C^{14}$ for both $P_1$ and $P_2$ from DNA labeled with tritium on the thymine methyl group and with $C^{14}$ in the thymine ring—suggests that their formation somehow involves removal of thymine methyl hydrogen. If either $P_1$ or $P_2$ should form *directly*, then neither could be cyclobutyl dimers, since the formation of such products would concern only the 5,6-double bonds, and would not affect the thymine methyl group at all. On the other hand, if both products are homogeneous and indeed cyclobutyl dimers, then they could not be formed directly, but rather through some intermediate photoproduct.

This argument is further supported by the fact that in frozen solution, where conditions are actually favorable for direct formation of T=T,[11] no such reduction in $H^3/C^{14}$ is observed for the major product $PT_2$ (i.e., T=T). Since thymine methyl hydrogen is lost during formation of the minor product $PT_1$, some kind of reaction involving the 5-$CH_3$ group occurs even when direct dimerization is favored. The fact that this minor product is converted by both hot acid and UV radiation to a product chromatographically equivalent to $PT_2$ suggests that $PT_2$ can be formed indirectly, and therefore that the same might be true for $P_2$. The possibility is also raised that $P_2$, as finally isolated, may be derived from some other stable preliminary photoproduct in the course of the acid hydrolysis of the DNA.

Although it is tempting to consider photooxidation products, such as those described in the introduction, as possible candidates for such an intermediate(s), it would be premature to speculate on their nature, especially since the influence of irradiation pH suggests that some ionic mechanism may be involved. It is nevertheless clear, however, that the manner in which the isolable photoproducts are formed is as relevant as their identity for understanding the biologically significant photochemistry of DNA.

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[1] Homodimer and heterodimer are used to denote those dimers consisting of like or unlike molecules, respectively. T=T, U=U, etc. are used to designate the cyclobutyl-type dimers which are linked by two bonds in order to avoid confusion with the single-bonded coupled product, T—T, U—U, etc.

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[18] The use of noncommittal designations in referring to observed products permits critical discussion of their identity without a confusion of terminology. It should be noted, however, that $P_1$ is described by other authors[5] as U=T, and $P_2$ as T=T. Detailed chemical characterization of $P_2$, isolated from irradiated DNA in milligram amounts will be reported elsewhere (Varghese, A. J., and S. Y. Wang, *Nature*, in press; another article has been submitted for publication).

[19] Donnellan and Setlow[4] have reported chromatographic separation of three photoproducts from irradiated *B. megatereum* spores, to which they refer as *a*, *b*, and *c*. They characterize *a* as U=T, but *b* and *c* remain uncharacterized. They have also obtained *b* and *c* from *E. coli* DNA irradiated *in vitro;* these two products, however, are very similar to the two peaks we observe, even from unirradiated samples, with respect to their $R_f$ values and per cent of the total thymine.

[20] Unless stated otherwise, all further chromatographic procedures used in characterizing $P_1$ and $P_2$ involved the use of solvent A.

[21] In these experiments, the methyl groups are monosubstituted (—$CH_2T$) since they were prepared by the reduction of —$CH_2OH$ with tritium. From the specific activity of the tritiated thymine (13.3 c/mole), it was calculated that 15% of the H atoms on the methyl group are tritium or that there are about equal numbers of —$CH_3$ and —$CH_2T$ in the population of thymine.

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