γ-IRRADIATION OF THYMINE DIMERS IN AQUEOUS SOLUTION

R. A. DEERING *and* WALLACE SNIPES

From the Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT Thymine dimers were irradiated in aqueous solution with ^{60}Co γ-rays in N_2 or O_2. Thymine and unidentified non-UV-absorbing products appeared. The thymine was identified by spectrophotometry, chromatography, and ability to support the growth of *Escherichia coli* 15 T^-. Residual dimer was determined by a UV-reversibility assay. The G-values for dimer breakage were approximately equal in N_2 and O_2. At low γ-doses, about two thymines were produced per dimer broken in N_2, whereas only about one thymine appeared per dimer broken in O_2. For dimer irradiated in frozen solution, the yield of thymine was at least 100 times less than in liquid.

INTRODUCTION

Ultraviolet light (UV) (200–300 mμ) irradiation of thymine in frozen solution, in dinucleotides, and in synthetic or natural polynucleotides produces thymine dimers (see reference 1 for a review). These dimers are important biologically because their production in DNA in vivo can lead to blockage of semiconservative DNA synthesis in those cells in which they are not removed by repair processes (2–4). The dimer predominantly produced by UV in frozen thymine solutions (5) or in DNA in liquid (6) is illustrated in Fig. 1. This dimer ($\widehat{TT}$) can also be monomerized by ultraviolet light but the wavelength dependence for this is different than for its formation (7, 8).

We have studied the effect of γ-irradiation of aqueous solutions of this dimer. These experiments indicate that this dimer is broken by ^{60}Co γ-rays to give thymine. The number of thymine molecules produced per dimer broken depends on whether the irradiation is performed on oxygenated or nitrogenated solutions. Some brief reports of related experiments by Myers and associates have appeared (9, 10).

MATERIALS AND METHODS

For dimer preparation, a 1% solution of thymine was frozen on dry ice in a shallow aluminum pan. This was irradiated for 3 hr with five 15-watt germicidal lamps at a distance of about 4 cm. The solution was then thawed, heated to 100°C, filtered, and left at room temperature overnight. The small, white, fragile crystals that appeared were collected by filtration and dried at 80°C. The absorbance of a test solution of this material was measured at 265 mμ

and from the known total concentration and the relative absorbances of thymine and thymine dimer, the approximate amount of each of these components was determined. All the precipitate was then dissolved in enough boiling water so that about 85% of the dimer would precipitate again when cooled to room temperature overnight, leaving the majority of the more soluble thymine in solution. The new precipitate was collected and dried. Following further absorbance determination of contaminating thymine, this procedure was repeated twice. At that time, no 265 mμ peak was observed in the spectrum, and later experiments indicated less than 1 part per 1000 of thymine in the preparation. The absorption spectrum (Fig. 2) of this preparation was the same as those previously published for thymine dimer (8, 11). The dimer prepared by this method should be the stereoisomer shown in Fig. 1 (5, 6). All dimer solutions for subsequent irradiations were prepared in Millipore-filtered distilled water, with no buffering. ^{14}C-labeled dimer was prepared by adding [$^{14}C_2$] thymine to cold thymine and isolating dimer as above.

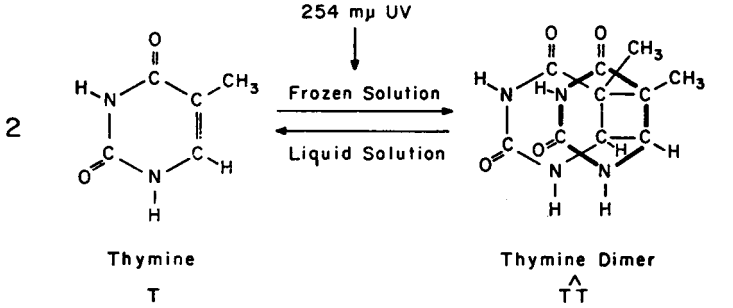

FIGURE 1 Formation and breakage of a thymine dimer by ultraviolet light, showing the structure of the thymine dimer used in these experiments.

^{60}Cobalt irradiations were done either in a Gammacell-100 or Gammacell-200 irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) at room temperature (23°C), at dose rates of 4.4 and 16.5 krad/min, respectively. These dose rates were determined from the manufacturer's specifications and have been verified in this laboratory with the Fricke dosimeter. No inconsistency in results due to these two different rates was observed. For short irradiations (up to 10 min in the Gammacell-100 or 3 min in the Gammacell-200), samples were pregassed in small vials with either nitrogen (N_2) for 5 min or oxygen (O_2) for 2 min, tightly stoppered, and irradiated immediately. No temperature control was necessary for these short irradiations. Since it was found that preoxygenated samples became anaerobic owing to radiation action for times longer than the above, samples to be irradiated for longer times were flushed continuously with either N_2 or O_2 through a small fritted-glass bubbler during irradiation. For the longer irradiations with the Gammacell-200, the sample temperature was held at 25°C by flowing water from a water bath through tubing surrounding the sample vial. The Gammacell-100 source did not appreciably increase the sample temperature above that of the room (~23°C). Except for the concentration-effect studies, all irradiations were done at a $\widehat{TT}$ concentration of 250 μg/ml in distilled water.

The amount of dimer remaining after γ-irradiation was determined by spectrophotometrically following to a maximum its reversal to thymine in solution (7, 8) during 254 mμ irradiation with germicidal lamps. Under our conditions this reversal was nearly quantitatively complete (Fig. 4).

Beckman DK-2A and DU-2 spectrophotometers (Beckman Instruments, Inc., Palo Alto, Calif.) were used for absorbance measurements.

Ascending chromatography of ^{14}C-dimer and ^{14}C-thymine was done on Whatman No. 1 paper with 80:12:30 *n*-butanol:acetec acid:water as the solvent (12). For all experiments, 5 μg of material with a specific activity of 10^{-3} μc/μg was applied. The R_f values were 0.29 and 0.64 for dimer and thymine, respectively. Following chromatography and air drying, strips were cut from the chromatograms and counted in Bray's solution (13) with a liquid scintillation counter.

Escherichia coli 15 T$^-$, a thymine-requiring bacterium originating with Barner and Cohen (14) was used for the microbial assay of thymine. Only thymine or thymidine has been shown to satisfy the nutritional requirements of this organism. Growth was in C-1 medium (15) supplemented with known amounts of thymine, unirradiated dimer, or γ-irradiated dimer. For routine growth, 5 μg thymine per ml was used. Cells to be used for the assays were grown to a concentration of 5 $\times$ 10^8/ml, cooled, collected on a 0.45 μ Millipore filter, washed with 30 ml of C-1 medium, and resuspended to a concentration of 1 $\times$ 10^8 per ml in C-1 medium. One ml of this suspension was added to each of several tubes containing 9 ml C-1 medium with known amounts of thymine or irradiated dimer. These tubes were then incubated at 37°C with aeration for various periods of time, and the turbidity at 625 mμ read with a Bausch & Lomb Spectronic 20 Colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

RESULTS

Spectrophotometric

Fig. 2 shows difference spectra (solid lines) for $\widehat{TT}$ irradiated in either N_2 or O_2 with 51 krad of γ-rays vs. unirradiated $\widehat{TT}$. For comparison, the absorption spectra vs. water for thymine at 10 μg/ml and $\widehat{TT}$ at 125 μg/ml are shown. There is good agreement between the spectrum of the chromophore produced by the γ-irradiation of

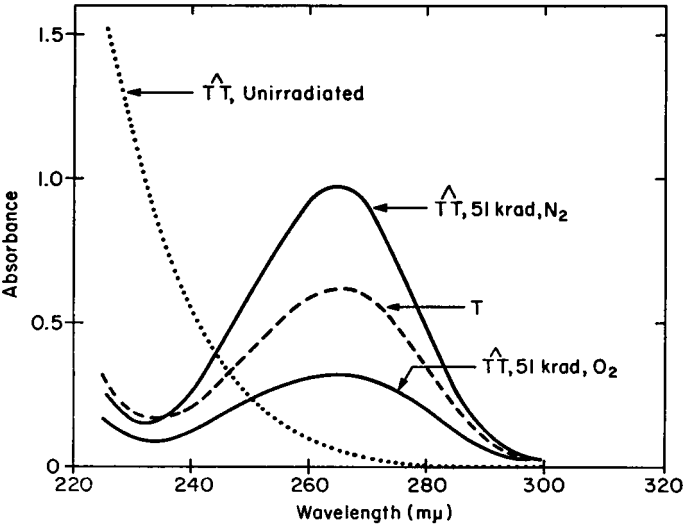

FIGURE 2 Solid lines: difference spectra for irradiated vs. unirradiated $\widehat{TT}$. Top: 51 krad γ-rays in N_2; bottom: 51 krad γ-rays in O_2. Samples were irradiated at a $\widehat{TT}$ concentration of 250 μg/ml, then diluted 2 times for recording of spectra. Dashed line: absorption spectrum of 10 μg thymine/ml vs. H_2O. Dotted line: absorption spectrum of 125 μg dimer/ml vs. H_2O.

$\widehat{TT}$ and that of thymine except for the relative magnitudes at about 233 mμ. This slight difference is expected since the absorbance loss due to converted $\widehat{TT}$ reduces the total absorbance compared to that of the unirradiated $\widehat{TT}$ in this region, thus making the difference spectra the net change for $\widehat{TT}$ lost and chromophore gained. If correction is made for this slight effect, agreement with the thymine absorption spectrum is improved. This correction is neglibible in the region around 260 mμ due to the very small absorbance of the $\widehat{TT}$ there. More chromophore appeared in the presence of N_2 than in O_2.

Chromatography

Chromatography of irradiated dimer showed a new spot with an R_f equal to that of thymine. Unirradiated dimer showed no spot at this position with the amounts used here. For 130 krad, 8.4% of the label was in this new spot for irradiation in O_2 and 18.4% for N_2. The remainder of the label appeared in the dimer position in both cases. A hint of another small spot was observed at the leading edge of the dimer region but was not resolved further by this chromatography technique. Higher resolution chromatography with this and other solvents would probably disclose other products of the radiolysis such as the material labeled "*X*" in later results.

Microbial Assay

Experiments were done to quantitate the growth of *E. coli* 15 T$^-$ on different known thymine concentrations and on irradiated $\widehat{TT}$. Fig. 3 is a plot of 625 mμ turbidity

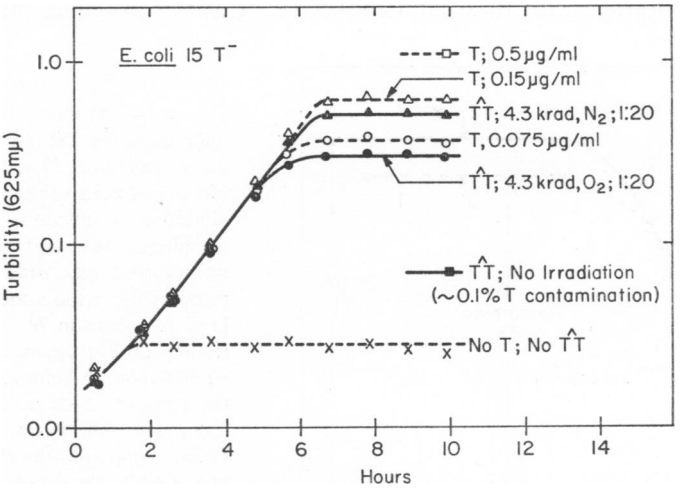

FIGURE 3 Growth curves of *E. coli* 15 T$^-$ for different control thymine concentrations (dashed lines) and unirradiated and irradiated $\widehat{TT}$ (solid lines). —□—, —△—, —○—, and —×— represent thymine concentrations of 0.5, 0.15, 0.075, and 0.00 μg/ml, respectively. —■— represents unirradiated $\widehat{TT}$ (12.5 μg/ml). —●— and —▲— represent $\widehat{TT}$ irradiated in O_2 and N_2, respectively. These irradiations were with 4.3 krad of γ-rays at a $\widehat{TT}$ concentration of 250 μg/ml, followed by a 20-fold dilution for the assay.

as a function of time for *E. coli* 15 T⁻ with different additives. The dashed lines are growth curves for known thymine concentrations, ranging from 0–0.5 μg/ml. The solid curves are for irradiated $\widehat{TT}$ (4.3 krad) in N_2 (▲) or O_2 (●) and unirradiated $\widehat{TT}$ (■). The latter gave very slight growth due to about 1 part per 1000 of contaminating thymine. The irradiated samples were diluted 20 times for the assay. It is clear that irradiated $\widehat{TT}$ satisfied the thymine requirement of *E. coli* 15 T⁻ and that unirradiated $\widehat{TT}$ did not. In addition, the assay indicated that more growth occurred on $\widehat{TT}$ irradiated in N_2 than in O_2. Experiments showed that unirradiated $\widehat{TT}$ at concentrations up to 25 μg/ml did not alter growth in the presence of added-thymine, except for a very slight stimulation due to the small amount of contaminating thymine.

Breakage of $\widehat{TT}$ to thymine and Unknown Substance(s) X

From the results presented in the three previous sections, the UV-absorbing chromophore resulting from the γ-irradiation of $\widehat{TT}$ in aqueous solution is identified as thymine (T). In order to determine how much $\widehat{TT}$ remained after various γ-doses, the reversal of $\widehat{TT}$ to thymine in solution by UV light was utilized. Fig. 4 shows some results for the UV reversal of a non-γ–irradiated $\widehat{TT}$ control (25 μg/ml) and a sample that had received 87 krad of γ-rays at a concentration of 250 μg/ml followed by a 10-fold dilution before the UV reversal. For 254 mμ UV exposures of 12 min or longer, the unirradiated $\widehat{TT}$ appeared to be quantitatively converted to T, since 25 μg/ml T should have an absorbance of 1.56 under these conditions, and an absorbance of 1.55 appeared for maximum conversion. For this example, the absorbance

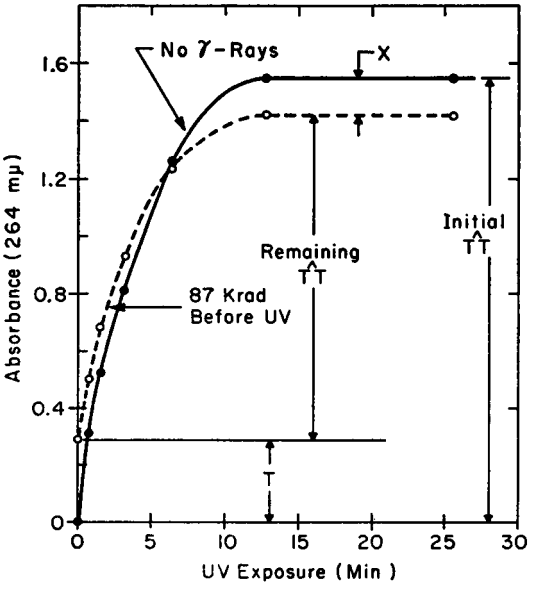

FIGURE 4 Monomerization of thymine dimer by 254 mμ UV subsequent to γ-irradiation. The absorbance at 264 mμ of a $\widehat{TT}$ solution (25 μg/ml) is plotted as a function of relative dose (minutes). —●— and —○— represent unirradiated and irradiated solutions respectively. Irradiation was with 87 krad of γ-rays in N_2 at a $\widehat{TT}$ concentration of 250 μg/ml, followed by a 10-fold dilution before reversal. "T" is the amount of thymine produced by the γ rays. "*X*" is the amount of unknown non-UV-absorbing products produced by the γ-rays. "Initial $\widehat{TT}$" is the amount of thymine dimer monomerized by UV in the sample receiving no γ-rays. "Remaining $\widehat{TT}$" is the amount of UV-reversible thymine dimer remaining after the γ-irradiation.

of the γ-irradiated sample was 0.29 for no UV exposure. This indicates the amount of thymine produced by the γ-rays. The dashed line shows the course of the conversion of the remaining $\widehat{TT}$ of this sample to thymine. The sum of the amount of thymine produced by the γ-rays and that produced by UV conversion did not equal the amount of thymine that would have appeared following complete UV conversion in a non-irradiated sample. The difference, labeled *X*, is therefore γ-ray-produced material that is neither a UV chromophore (identified as thymine) nor $\widehat{TT}$ (as measured by degree of UV conversion to thymine). *X* may be more than one substance, but further information on its composition is not currently available. From the results of the microbial assays, all of the γ-ray-produced 264 mμ chromophore appears to be thymine.

Amounts of $\widehat{TT}$, T, and X as a Function of Gamma Dose

Fig. 5 indicates the amounts of remaining $\widehat{TT}$, and amounts of T and *X* produced in terms of per cent total solute mass, as a function of γ-ray doses up to 1 Mrad for N_2 and O_2. These data were determined spectrophotometrically by the method illustrated in Fig. 4. All samples were irradiated at $\widehat{TT}$ concentrations of 250 μg/ml. The rates of loss of $\widehat{TT}$ for N_2 and O_2 were approximately the same, although they varied somewhat in details. The amount of T produced in N_2 was considerably higher than in O_2. The amount of *X* produced was higher in O_2. For both N_2 and O_2, the amount of T increased to a maximum and then decreased. This decrease was probably due to the radiolysis of T at the higher γ-ray doses. A detailed theoretical analysis of these curves over the complete dose range shown here is not easily done since the degree of competition of various products (T and *X*) for radiation-produced radicals and the detailed mechanisms of the radical action are not known. The lower

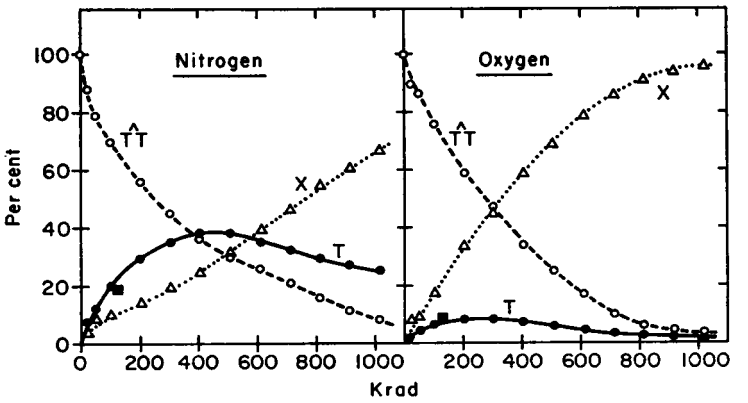

FIGURE 5 Percentage of thymine dimer ($\widehat{TT}$), thymine (T), and unknown product (*X*) as a function of γ-ray dose in N_2 and O_2. All measurements are spectrophotometric except ■, which is from chromatography.

maximum value reached by the thymine in O_2 as compared to N_2 may be due to a greater rate of thymine breakdown in O_2, a result consistent with the literature (16, 17). In the presence of N_2, the amount of thymine produced reached almost 40% of the initial amount of $\widehat{TT}$. Two experimental points, one for N_2 and one for O_2, each indicated by ■, were determined from the chromatography experiments and agree well with the spectrophotometric results.

In order to more accurately determine the rates of production of T in the low dose regions of these curves, further spectrophotometric and microbial assays were done in the dose range of 250 rad to 50 krad. The microbial assay was usable for T productions as low as 0.03%, corresponding to doses down to 250 rad. The spectrophotometric assay could be used down to 0.5% T and 4000 rad. The samples were

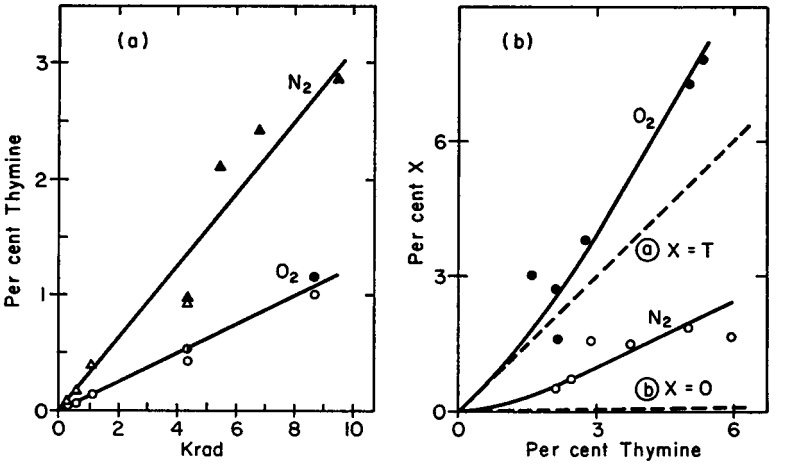

FIGURE 6 (*a*) Per cent thymine produced at low γ-ray doses in N_2 and O_2. ▲, irradiated in N_2, spectrophotometric assay; △, irradiated in N_2, microbial assay; ●, irradiated in O_2, spectrophotometric assay; ○, irradiated in O_2, microbial assay. (*b*) Per cent unknown product (*X*) as a function of per cent thymine produced (T). —●— and —○— represent irradiations in O_2 and N_2, respectively. ⓐ is the response expected for [*X*] = [T], ⓑ for [*X*] = 0.

irradiated at 250 μg $\widehat{TT}$ per ml and then diluted appropriately for assay if necessary. Some results from these low dose experiments are presented in Fig. 6 *a*. In agreement with the results obtained at the higher doses, the initial rate of T production was higher in N_2 than in O_2, even at γ-ray doses down to 250 rad and amounts of T less than 0.1%. The initial slope for T production in N_2 was 2.5 times that in O_2. The microbial assay and spectrophotometric assays agreed, indicating that all of the UV chromophore was thymine.

In order to obtain estimates of the amount of *X* at low doses, some additional experiments were done similar to those of Fig. 4, with very small amount of T and *X*. These required special care in adjustment of concentration, prevention of evaporation, and absorption measurements. Even then, considerable scatter was ob-

tained. Various γ-ray doses were given to obtain small amounts of thymine, and the amount of X was determined for each point. The amount of X as a function of the amount of T is plotted in Fig. 6 *b* for N_2 and O_2. If one desires, the remaining amount of $\widehat{TT}$ can be obtained by adding T and X and subtracting from initial $\widehat{TT}$. The dashed line (*a*) would be expected for $[X] = [T]$ and (*b*) for $[X] = 0$. The amount of X relative to T produced in O_2 was much higher than in N_2. The scatter precludes a definitive analysis of these results. However, they indicate that about 0.7–1.0 T's were produced per $\widehat{TT}$ lost for O_2 and about 1.6–2.0 T's appeared per $\widehat{TT}$ lost for N_2. It is not inconsistent with these results that at very low doses (below about 1% thymine on Fig. 6 *b*) there were two T's produced per $\widehat{TT}$ broken in N_2 and 1 X + 1 T per $\widehat{TT}$ in O_2.

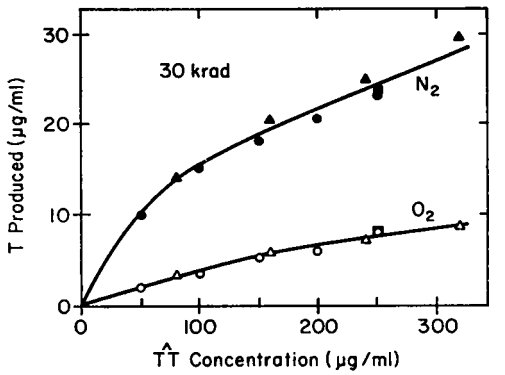

FIGURE 7 Effect of thymine dimer concentration during irradiation on yield of thymine for irradiation in N_2 (closed symbols) and O_2 (open symbols). The γ-ray dose was 30 krad. Different symbols are for different experiments.

All previous results were for an initial concentration of 250 μg $\widehat{TT}$ per ml in distilled water during irradiation. This concentration was used routinely in most experiments since the solubility of $\widehat{TT}$ was only about 400 μg/ml at room temperature. The amount of T produced by 30 krad as a function of $\widehat{TT}$ concentration up to 320 μg/ml for N_2 or O_2 is shown in Fig. 7. The curves for irradiation in N_2 or O_2 have a decreasing slope at the higher concentrations as expected for radical action on a solute. However, the curves do not really plateau at concentrations almost to the limit of solubility. The ratio of T produced in N_2 to that produced in O_2 increased somewhat as the $\widehat{TT}$ concentration was lowered. The yields may be approaching a constant value at high $\widehat{TT}$ concentrations but this cannot be ascertained owing to the low solubility of $\widehat{TT}$. In any case, these curves are consistent with radical action rather than "direct" effect of the irradiation.

The rate of T production was reduced at least 100 times if the solutions were irradiated frozen at temperatures of -5°C or below.

Since all irradiations were done in unbuffered distilled water to eliminate solute effects, it was of interest to determine the pH during the course of the irradiation. $\widehat{TT}$ solutions (250 μg/ml) with either O_2 or N_2 bubbling were irradiated and the pH determined after various doses. The initial pH was about 6 for both cases. A dose

of 720 krad in N_2 or O_2 dropped the pH to about 4.6. In the low dose range (<30 krad) used to determine our G-values, the change from unirradiated solutions was less than 0.3 pH units.

DISCUSSION

γ-irradiation of thymine dimers in aqueous solution produces thymine and other products. G-values for thymine production have been determined in the low dose region where as little as 0.1% of the $\widehat{TT}$ has been degraded. These G-values are 6.1 ± 0.2 in N_2 and 2.6 ± 0.2 in O_2. Since $\widehat{TT}$ amounts only slightly different from 100% were not easily measurable and the amounts of X were not determined directly, the G-values for $\widehat{TT}$ breakage and X appearance are only approximate. However, the results tentatively indicate that at low doses 1.6–2.0 T's are produced per $\widehat{TT}$ broken in N_2 and 0.7–1.0 T's are produced per $\widehat{TT}$ broken in O_2. These numbers lead to initial G-values for $\widehat{TT}$ breakage of 3.0–3.8 in N_2 and 2.6–3.7 in O_2. Initial G-values for X appearance would then be 0–1.5 in N_2 and 2.6–4.7 in O_2. The G-values for $\widehat{TT}$ breakage at low dose in N_2 and O_2 may actually be equal since the ranges of possible values overlap. Even though it is probably an oversimplification, in the subsequent discussion, we will assume that 2 T's appear per $\widehat{TT}$ broken in N_2 and that 1 T + 1 X appear per $\widehat{TT}$ broken in O_2. Our results are consistent with this possibility. At present this statement must be limited to a $\widehat{TT}$ concentration of of 250 μg/ml. The dependence of thymine production on $\widehat{TT}$ concentration is not straightforward (Fig. 7) in that the ratio of thymine produced in N_2 to that produced in O_2 is not a constant when the $\widehat{TT}$ concentration is changed.

As soon as thymine is produced from the breakage of $\widehat{TT}$, some of this will be acted on by the γ-ray–produced radicals to give other products. Hence, during the irradiations depicted in Fig. 5, competing processes of thymine production and degradation are occurring. The rate of thymine degradation will depend on its concentration and its ability to compete with $\widehat{TT}$ and other products for radicals. The maxima in the amounts of T can be interpreted as occurring at that point where the rates of T appearance and radiolysis are equal. For higher doses, the rate of radiolysis of T exceeds its rate of appearance and the amount of T decreases. Our results are consistent with thymine degradation being more rapid in O_2 than in N_2. There is little detailed information in the literature on rates of thymine degradation in N_2 and O_2, particularly at low thymine concentrations and in the presence of other solutes; however, ratios of rates of degradation in O_2 as compared to N_2 have been reported to be in the range of 2–3 (16, 17). A detailed analysis of the higher dose regions of Fig. 7 has not been attempted due to lack of sufficient information, such as G-values for thymine destruction in N_2 and O_2 at various concentrations, effects of changing pH, and relative competing abilities of different materials such as $\widehat{TT}$, T, and X for radicals.

Fig. 8 shows a simplified interpretation of our results for the initial degradation of $\widehat{TT}$ at low γ-ray doses. The degree of γ-degradation of T and X should be very small in this region since the very large amounts of $\widehat{TT}$ relative to T and X should allow the $\widehat{TT}$ to remove essentially all the radicals from solution. This simplified scheme depicts $\widehat{TT}$ radical ($\widehat{TT}\cdot$) production in either N_2 or O_2 with subsequent breakage to T and a T radical (T·). In N_2, the T· goes to T giving a total of 2 T's per $\widehat{TT}$. In O_2, the T · reacts with O_2 to go to X, thus giving one T and one X per $\widehat{TT}$. It would be premature on the basis of present evidence to speculate too much on the details of this proposed reaction; however, a little speculation may be useful. We have found that Fenton's reagent (OH ·) will produce thymine from $\widehat{TT}$. This leads to the possibility that it is γ-ray–produced hydroxyl radicals that are at least partially responsible for the initial $\widehat{TT}$ radical. The product(s) X may be peroxides and/or glycols, species commonly observed following γ-irradiation of thymine solutions (16, 17).

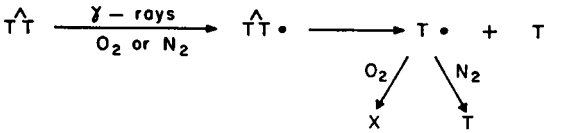

FIGURE 8 Simplified scheme for thymine dimer breakage in N_2 and O_2.

The quantum yield for $\widehat{TT}$ monomerization by UV is approximately unity (8). The question arises as to whether short wavelength UV in amounts sufficient to give the observed dimer breakage could be produced by γ-rays in the $\widehat{TT}$ solution from Cerenkov radiation and from emission by excited water molecules or radicals. From the work of Sitharamarao and Duncan (18) the number of photons produced per 100 ev absorbed in the solution, in the wavelength region of 180–230 mμ, should not exceed 0.03 for ^{60}Co γ-rays. Since we have observed that approximately three $\widehat{TT}$ molecules are broken per 100 ev absorbed, we conclude that $\widehat{TT}$ breakage by UV light is insignificant in the γ-radiolysis of dimer solutions.

We do not know how effectively γ-rays break dimers in DNA. However, based on our experiments, it is reasonable to assume that the γ-breakage of a large enough number of UV-produced dimers to bring about a partial reversal of UV damage and hence biological recovery would require large γ-ray doses. These would probably be so large that the damaging effects of the γ-rays on other parts of the DNA would far outweigh any advantage due to dimer breakage.

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