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Malondialdehyde precursors in gamma-irradiated DNA, deoxynucleotides and deoxynucleosides

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ABSTRACT

Gamma-irradiation of DNA, deoxynucleosides, or deoxynucleotides produces material that reacts with thiobarbituric acid to form a chromophore with maximum absorbance at 532 nm. This material is not malondialdehyde. We have identified a new radiation product (thymin-1'-yl)-propenal as the TBA-reactive product of gamma-irradiation of thymidine. Thymine-propenal has been described by other investigators as a product of bleomycin-treatment of DNA. Irradiation of thymidine nucleotides produces phosphorylated precursors to thymine-propenal. Studies of the requirements for formation of TBA-reactivity indicate a mechanism involving reaction of a free radical with the deoxyribose moiety and molecular oxygen.

On the basis of these results it is proposed that gamma-irradiation produces TBA-reactive material in DNA by the same reaction sequence in which bleomycin catalyzes the formation of base-propenals in DNA. Bleomycin and gamma-irradiation differ in the extent to which the sequence proceeds to completion with release of free base-propenals.

INTRODUCTION

Following exposure to ionizing radiation, a variety of alterations are present in DNA. Both alterations in the nucleotide bases and in the deoxyribose-phosphodiester backbone of the DNA occur and for many of these lesions the chemical structure has been determined (1,2). Knowledge of the structure of such alterations in DNA can be useful in determining the exact role of each of these alterations in the cytotoxic, mutagenic and carcinogenic effects of ionizing radiation.

The chemical structure has not been determined for all ionizing radiation-induced products in DNA. One such product of gamma-irradiation of DNA or deoxynucleotides is a material which is like malondialdehyde in its ability to react with thiobarbituric acid (TBA) to form a chromophore with strong absorbance at 532 nm (1,3-5). Formation of this chromophore is not completely specific for malondialdehyde. Compounds such as oxidized lipids (6) and the base-propenals produced by bleomycin treatment of DNA (7,8) are also positive in this assay, reacting with TBA to form the same chromophore as

is formed by reaction with malondialdehyde. Kapp and Smith (4) suggested that the TBA-reactive material in solutions of gamma-irradiated DNA was not malondialdehyde because it precipitated in ethanol with DNA, whereas malondialdehyde itself did not.

The experiments reported here were performed to determine the nature of the malondialdehyde-like material in gamma-irradiated DNA. To reduce the complexity of this analysis, we have also used deoxynucleosides and deoxynucleotides as model targets for gamma-irradiation.

MATERIALS AND METHODS

Materials. Deoxythymidine, thymine, cytosine arabinoside, cytidine, ribose, 2'-deoxyribose, 2-thiobarbituric acid and calf thymus deoxyribonucleic acid were purchased from Sigma Chemical Company (St. Louis, MO). Deoxyadenosine, deoxycytidine, 2',3'-dideoxythmidine, 3' thymidine monophosphate, 5' thymidine monophosphate, 3',5'-thymidine diphosphate, and 3',5'-deoxyadenosine diposphate were purchased from P. L. Biochemicals (Milwaukee, WI). Blenoxane, the clinically used mixture of bleomycins A and B_2, was obtained from Bristol Laboratories. Micrococcal nuclease was obtained from Worthington Diagnostics and calf alkaline phosphatase was obtained from Boehringer Mannheim. Malondialdehyde was generated from malondialdehyde-bis-(dimethyl acetal) as described by Burger et al., (7).

Irradiation. Gamma-irradiation was delivered at 25°C by a Gammarad Irradiator Model GR9 at a dose rate of 50 rad/sec. Unless otherwise noted, samples were irradiated in distilled deionized water and ambient atmosphere. Where indicated, solutions were equilibrated with either oxygen, oxygen-free nitrogen or nitrous oxide for 15 minutes prior to and during irradiation. Radiation chemical yields are expressed as G values where one G-unit represents 1 molecule of product formed for 100 eV of energy absorbed. One G-unit also represents 1.04 uM of product formed per 1000 rad in aqueous solution.

Chromatography. HPLC equipment included a Spectra-Physics solvent delivery system with Valco injector and a variable wave-length detector (ISCO, Lincoln NB). An Econosphere (5 micron particles, 25 cm x 4.6 mm) silica column (Alltech Associates, Deerfield, IL) was used for purification and analysis of thymine-propenal with 100% acetonitrile as the mobile phase. A 5 micron particle (25 cm x 4.6 mm) Amine column (Alltech Associates) was used for resolution of the irradiation products of thymidine nucleotides. Flow rates were 1.0 ml/min at ambient temperature. TLC was performed on silica gel plates (0.1 mm thickness, 254 nm fluorescent indicator, Baker Chemicals) with

solvent systems I (ethyl acetate:isopropanol:water 74:19:7), II (chloroform:isobutanol, 75:25), or III (chloroform:isobutanol 90:10). TBA-reactive products were visualized as pink spots after spraying plates with 0.6% TBA and heating at 80°C for 10 min.

Preparation of Compound (I) from Gamma-Irradiated Thymidine. Thymidine (0.5 mM) in distilled deionized water (400 ml) was irradiated to 40 krads. Following irradiation, the sample was reduced to 1 ml *in vacuo* at 40°C. The sample was then applied to a preparative silica gel TLC plate (Brinkmann Instruments, Sil G-100, 1.0 mm thickness), dried under a stream of cool air and chromatographed with solvent system II. When the solvent front reached 5 cm from the origin, the plate was dried. TBA-reactive material (Compound I) migrated at R_f .80-.85 (thymine R_f = .60, thymidine R_f = .48) and was eluted from the plate with acetonitrile. After concentration to 0.5 ml by lyophilization, the sample was subjected to silica gel HPLC (detection at 300 nm). A single peak of UV absorbance (300 nm) and thiobarbituric acid-reactivity eluted at 5.4 min (compound I). Rechromatography of purified compound I by silica gel HPLC with monitoring at 300 nm or 260 nm revealed only a single UV absorbing peak coincident with the TBA-reactivity. In addition, analysis of purified compound I by silica gel TLC (Systems I and III) revealed only a single UV absorbing and TBA-reactive spot which comigrate. Compound I is stable for at least one month in acetonitrile at -20°C or at -70°C in H_2O for 6 months.

Assays and Enzyme Treatment. Malondialdehyde or malondialdehyde equivalents were determined as described by Burger et al. (7). Nucleotides were quantitated by absorbance at 260 nm. For Micrococcal nuclease digestion of DNA, reaction mixtures (250 ul) contained 3 umol gamma-irradiated DNA (1.5 nmol malondialdehyde equivalents), 7.5 umol calcium chloride, 30 mM sodium formate (pH 8.8) and 1 U Micrococcal nuclease. Following incubation at 37°C for the indicated time, reactions were terminated by the addition of sodium acetate to 0.15 M and two volumes of cold ethanol. Following centrifugation (10,000 Xg, 5 min), nucleotides and malondialdehyde equivalents were quantitated in the supernatant and pellet as described above. For alkaline phosphatase treatment, reaction mixtures (180 ul) contained 0.5 M Tris-HCl (pH 8.0), 0 - 12.5 umol of nucleotide and 0.4 U of calf alkaline phosphatase. Incubation was at 37°C for 5 h. Following phosphatase treatment, samples to be analyzed by Amine column HPLC were injected directly. Samples to be analyzed by silica gel HPLC were first mixed with an equal volume of

acetonitrile : methanol (95:5) and rendered anhydrous by the addition of solid magnesium sulfate.

Preparation of [^{3}H]-Labeled Thymine-Propenal. Reaction mixtures (100 ul) for the production of [^{3}H] thymine-propenal contained [^{3}H-methyl-thymidine]-labeled Hela cell DNA (50 ug, 10^7 cpm), 230 uM Blenoxane, 230 uM ferrous amonium sulfate and 50 mM Tris-HCl (pH 7.5). Following 30 min incubation at 0°C, [^{3}H]-thymine-propenal was isolated as described by Giloni et al., (8).

RESULTS

Requirements for the Production of TBA-Reactive Material. To elucidate the mechanism by which gamma-irradiation produces TBA-reactive material in DNA, we have investigated the relationship between chemical structure and production of TBA-reactive material. TBA-reactive material was quantitated following irradiation, under aerobic conditions, of DNA or a series of compounds that are either DNA components or analogues of DNA components (Table 1). For each of these compounds the amount of TBA-reactive material increased linearly with irradiation dose over the range 0-100,000 rad and the amount of TBA-reactive material produced is independent of the concentration of substrate over the range 0.2-1.0 mM. This is consistent with the production of TBA-reactive material by a mechanism secondary to radiolysis of water. The yield of TBA-reactive material for each substrate is reported as its G value (see Methods) in Table 1. The yield of TBA-reactive material produced by gamma-irradiation of DNA or thymidine nucleotides agrees well with the yield reported by other investigators (1,3).

Comparison of the yields of TBA-reactive material from various irradiation substrates demonstrates that the presence of a deoxyribose moiety is required for the production of TBA-reactive material on gamma-irradiation. In the absence of a deoxyribose moiety (thymine, adenine), or in the presence of a deoxyribose altered by either the addition of a 2′ hydroxyl group (ribose, cytidine, cytosine arabinoside) or the absence of a 3′ hydroxyl group (2′,3′ dideoxythymidine) TBA-reactive material is not formed.

Among the deoxyribose-containing compounds there are quantitative differences in the amount of TBA-reactive material produced by irradiation. While production of TBA-reactive material occurs on irradiation of deoxynucleosides or deoxynucleotides containing either thymine, cytosine or adenine bases, the yield is higher when an adenine base is present. TBA-reactive material is present following irradiation of either

TABLE I
Production of Thiobarbituric Acid-Reactive Material by Gamma-Irradiation of Nucleic Acid Components.

Compound	G Value	
	Concentration of Irradiation Substrate	
	0.2 mM	1.0 mM
SUGAR		
ribose	<0.01	<0.01
deoxyribose	0.26 ± 0.06	0.38 ± 0.09
BASE		
thymine	<0.01	<0.01
adenine	< 0.01	<0.01
NUCLEOSIDE		
thymidine	0.11 ± 0.03	0.09 ± 0.03
deoxyadenosine	0.34 ± 0.04	0.44 ± 0.06
deoxycytidine	0.13 ± 0.02	0.13 ± 0.02
cytidine	0.02 ± 0.01	0.01 ± 0.01
cytosine arabinoside	<0.01	<0.01
2',3'-dideoxythymidine	< 0.01	N.D.
NUCLEOTIDE		
3'-dTMP	0.10 ± 0.02	N.D.
5'-dTMP	0.10 ± 0.01	N.D.
3',5'-dTDP	0.06 ± 0.01	0.05 ± 0.01
3',5'-dADP	0.11 ± 0.01	0.15 ± 0.02
DNA		
double-stranded	0.11 ± 0.03	0.17 ± 0.03
single-stranded	0.06 ± 0.01	0.10 ± 0.01

The indicated compounds in water and equilibrated in air were gamma-irradiated to doses of 0-40 krad. One G-unit equals 1.05 μM malondialdehyde equivalents formed per krad absorbed dose. Results are expressed as the average ± one standard deviation. N.D. = not determined.

single-stranded or double-stranded DNA but the yield is reduced for single-stranded DNA. Both of these observations are consistent with a model in which TBA-reactive material is produced in a reaction of free radicals with the deoxyribose moiety of deoxynucleosides, deoxynucleotides or DNA. The pyrimidine bases can compete with the deoxyribose moiety for reaction with free radicals to a greater extent than the purine bases (1). Likewise, the increased yield of TBA-reactivity following irradiation of double-stranded DNA versus single-stranded DNA is explained by the greater exposure of the bases in single-standed DNA to free radical attack and concomitant competition for

TABLE II
Inhibition of Formation of Thiobarbituric Acid-Reactive Material from Thymidine by Free Radical Scavengers.

SCAVENGER	G value	(%)
None	0.113	100
Ethanol	0.039	34.5
n-Butanol	0.023	20.4
Dimethylsulfoxide	0.020	17.7
Glycerol	0.019	16.8
beta-Mercaptoethanol	0.010	8.8

Solutions of thymidine (0.2 mM) and the additions shown (1.0 mM) were irradiated to 50 krad in air and TBA-reactivity determined as described in Methods.

the free radicals that produce TBA-reactive material.

Free Radical Scavengers Inhibit Gamma-Ray Production of TBA-Reactive Material. To test further whether hydroxyl free radicals might be involved in production of malondialdehyde-like material, thymidine was irradiated in air in the presence of the hydroxyl radical scavengers glycerol, dimethylsulfoxide, n-butanol, ethanol, or beta-merceptoethanol (1). The presence of these compounds during irradiation all produced a marked decrease in the yield of TBA-reactive material (Table 2). Similar inhibition of production of TBA-reactive material was observed with other irradiation substrates in the presence of free radical scavengers (data not shown). These results are also consistent with a requirement for free radicals in the production of TBA-reactive material.

Role of Oxygen in the Production of TBA-reactive Material. It has been reported (9) that molecular oxygen is required for the production of the TBA-reactive base-propenals during bleomycin treatment of DNA. We have investigated the role of oxygen in production of TBA-reactive material by gamma-irradiation. Solutions of DNA, nucleotides or nucleosides were equilibrated with either air, oxygen or oxygen-free nitrogen prior to and during gamma-irradiation (Table 3). The production of TBA-reactive material was found to be dependent on the presence of oxygen for the substrates DNA, 3',5'dTDP and 5'dTMP. However, irradiation of compounds that do not contain

TABLE III
Formation of Thiobarbituric Acid-Reactive Material in Irradiated Solutions Equilibrated with Air, Oxygen, Nitrogen or Nitrous Oxide.

COMPOUND	ATMOSPHERE			
	Air	Oxygen	Nitrogen	Nitrous Oxide
DNA (1.0 mM)	0.13 ± 0.06	0.16 ± 0.03	0.03 ± 0.01*	N.D.
DNA (0.2 mM)	0.11 ± 0.01	0.09 ± 0.02	0.03 ± 0.01*	N.D.
3',5'TDP (1.0 mM)	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.01*	0.02 ± 0.01*
3',5'TDP (0.2 mM)	0.06 ± 0.01	0.06 ± 0.01	0.02 ± 0.00*	0.02 ± 0.00*
5'TMP (0.2 mM)	0.05 ± 0.01	0.06 ± 0.01	0.01 ± 0.00*	N.D.
3'TMP (0.2 mM)	0.07 ± 0.03	0.06 ± 0.01	0.07 ± 0.03	N.D.
Thymidine (0.2 mM)	0.11 ± 0.03	0.06 ± 0.02*	0.10 ± 0.03	0.15 ± 0.02*

Samples (1.0 ml) at the indicated concentrations were equilibrated with air or the indicated gas, irradiated to 50 krad, and the TBA-reactvity determined as described inMethods. The results marked by asterisks differ significantly ($p < 0.05$) from the G value for irradiation in air. Results are expressed as the average ± one standard deviation of at least three determinations.

5′ phosphate esters such as 3′dTMP or thymidine produces essentially equal amounts of TBA-reactive material in the presence or absence of oxygen.

We have also investigated the effect of the presence of N_2O during gamma-irradiation on the production of TBA-reactive material. Solutions purged with N_2O differ from ambient atmosphere in two ways. Oxygen is removed and N_2O scavenges aqueous electrons converting them to hydroxyl radicals (1). Irradiation of 3′,5′dTDP under N_2O results in substantially less TBA-reactive material than irradiation under air. We attribute this reduction to the requirement for oxygen in production of TBA-reactive material from 3′,5′dTDP. However, the amount of TBA-reactive material produced by irradiation of thymidine under N_2O is significantly increased over that produced under air. As production of TBA-reactive material from thymidine does not require oxygen, we attribute this increase to the conversion of electrons to hydroxyl radicals by N_2O.

The Thiobarbituric Acid-Reactive Products of Gamma-Irradiation. The absorbance spectra (470-580 nm) of the chromophores formed by the reaction of

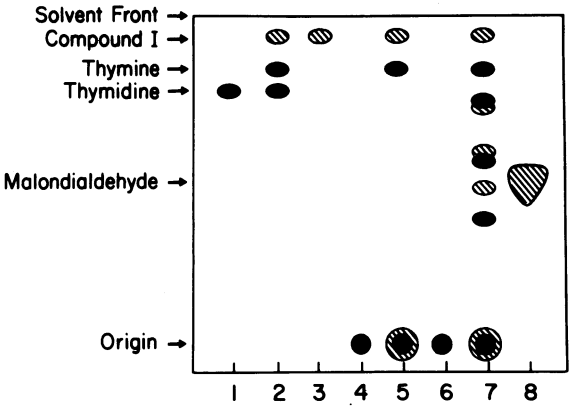

Figure 1. Analysis of Gamma-Irradiated Thymidine, 3' Thymidine-Monophosphate or DNA by Silica Gel Thin-Layer Chromatography. Samples were lyophilized to 50 ul and resolved by silica gel TLC in System I. Lane 3 and lane 8 contained 2 nmoles of purified compound I (thymine-propenal) or malondialdehyde respectively, prepared as described in Methods. The other samples (1.5 ml, 0.5 mM as nucleoside) were; lanes 1,2 thymidine, lanes 4,5 3'dTMP and Lanes 6,7 calf thymus DNA. Samples in lanes 2,5 and 7 were gamma-irradiated (10 krad) prior to lyophilization. UV-absorbing compounds were located under short-wave UV illumination (solid areas) and TBA-reactive compounds (hatched areas) were located as described in Methods.

TBA with the irradiation products of thymidine, thymidine nucleotides or DNA are all identical to that produced by reaction of TBA with malondialdehyde (data not shown). However, this is not proof that malondialdehyde is present. Giloni and coworkers (8) have reported that reaction of base-propenals with TBA produces the same chromophore as reaction with malondialdehyde.

The Thiobarbituric Acid-Reactive Product of Thymidine Irradiation. To determine the nature of the thiobarbituric acid-reactive material produced by irradiation of thymidine, we have separated the products of thymidine irradiation by silica gel TLC. At a dose of less than 10 krad no free malondialdehyde was detected and only a single TBA-reactive product (compound I) was visualized. This product has an R_f = 0.93 distinctly different from that of malondialdehyde R_f = 0.52 (Fig. 1). To study compound I further, it was purified from solutions of irradiated thymidine by preparative TLC on silica gel (R_f = 0.83, System II) followed by high pressure liquid chromatography. Purified compound I was homogenous as determined by a second chromatography on silica HPLC or two different TLC systems (Systems I and III, Materials and Methods). Compound I has the ultraviolet absorbance spectrum shown in Figure 2 with a maximum at 300 nm and ε_{300} = 30.7 $mM^{-1}cm^{-1}$ calculated

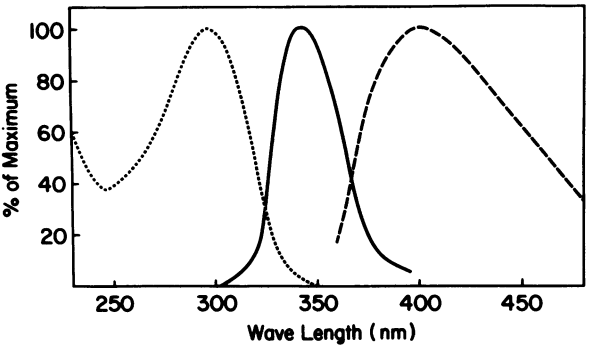

Figure 2. UV-Absorbance and Fluorescent Excitation and Emission Spectra of Compound I. Purified compound I was dissolved in water and the ultraviolet absorbance determined (dotted line). Fluorescence was determined in a Perkin-Elmer Model MDF-4 flouorescence spectrometer. Excitation spectrum for fluorescence (solid line) was determined with emission detection at 400 nm and emission spectrum (dashed line) was determined with excitation at 347 nm. Results are expressed as percent of maximum UV-absorbance or fluorescence.

on the assumption that one mole of compound I gave the same TBA-reactivity as one mole of malondialdehyde. Compound I is fluorescent with an excitation peak at 347 nm and an emission peak at 400 nm (Figure 2).

One of the TBA-reactive compounds in solutions of bleomycin-treated DNA has been demonstrated to be thymine-propenal (8). To determine if compound I is thymine-propenal, we have compared its chromatographic behavior to authentic thymine-propenal prepared by bleomycin treatment of DNA. Compound I, chromatographed on silica gel TLC in solvent System I, has a mobility relative to thymine (R_T = 1.2) identical to that described by Giloni and coworkers (7) for thymine-propenal. Compound I also comigrates with authentic ^{3}H-labeled thymine-propenal derived from bleomycin-treated DNA in two other TLC systems tested (System II, R_f = 0.83; System III, R_f = 0.75).

In acid solutions, thymine-propenal undergoes hydrolysis to thymine and malondialdehyde. When compound I is first treated for 10 minutes in 0.2 N HCl at 90°C, the amount of UV-absorbing and TBA-reactive material eluting at the position of compound I is reduced (Fig. 3). Hot-acid treated samples of compound I contain new UV-absorbing material, eluting at positions of authentic thymine and malondialdehyde and a new peak of TBA-reactive material at the elution position of malondialdehyde. The products of hot-acid treatment were further identified as thymine and malondialdehyde respectively by their UV-absorbance spectra. Quantitation by UV-absorbance demonstrated that 1.0 mole of thymine and 0.83 mole of malondialdehyde were recovered for

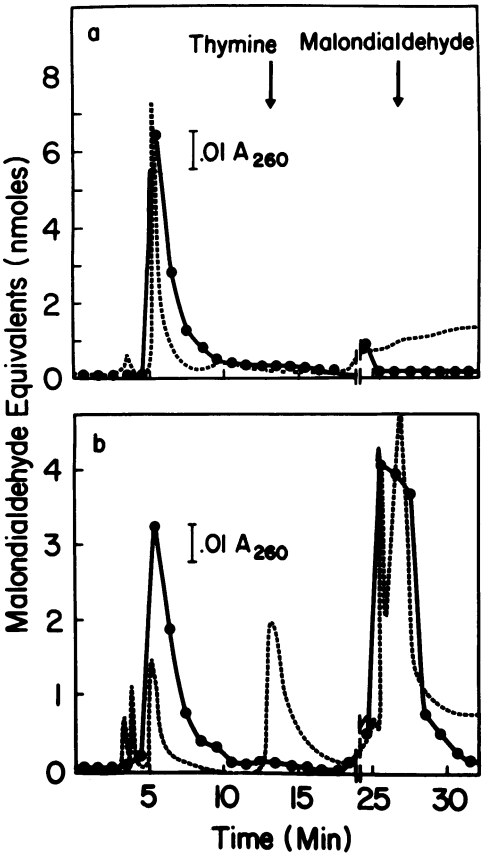

Figure 3. Products of Acid Hydrolysis of Compound I. Samples of (a) 14 nmoles of purified I or (b) 25 nmoles of purified I which had been heated to 90°C for 10 min. in 0.2 N HCl, were frozen, lyophilized and resuspended in 100 ul of acetonitrile. Samples were resolved by silica gel HPLC (mobile phase acetonitrile 100% 0-15 min., linear gradient to 100% methanol 15-25 min., flow rate 1 ml/min.). Eluted fractions were monitored at 260 nm (dotted line) and fractions were collected at 1 min intervals. Fractions were assayed for TBA-reactivity (closed circles) as described in Methods. The arrows indicate the time of elution of authentic thymine and malondialdehyde.

each mole of compound I destroyed by acid-heat treatment. From these data we conclude that the major TBA-reactive product of thymidine irradiation is thymine-propenal.

TBA-reactive Products of 3'dTMP Irradiation. To determine the nature of the TBA-reactive material produced by gamma-irradiation of nucleotides, irradiated solutions of 3'dTMP were resolved by silica gel TLC (Figure 1). No free malondialdehyde was detected. Gamma-irradiation produces small amounts of TBA-reactive material that was identified as thymine-propenal by its comigration with authentic thymine-propenal in three solvent systems. However, the predominant TBA-reactive product of 3'-dTMP irradiation was neither malondialdehyde nor thymine propenal but material that remained on the origin during silica gel TLC.

Phosphorylated Thiobarbituric Acid-Reactive Material in Irradiated Thymidine Nucleotides. The difference in chromatographic behavior between

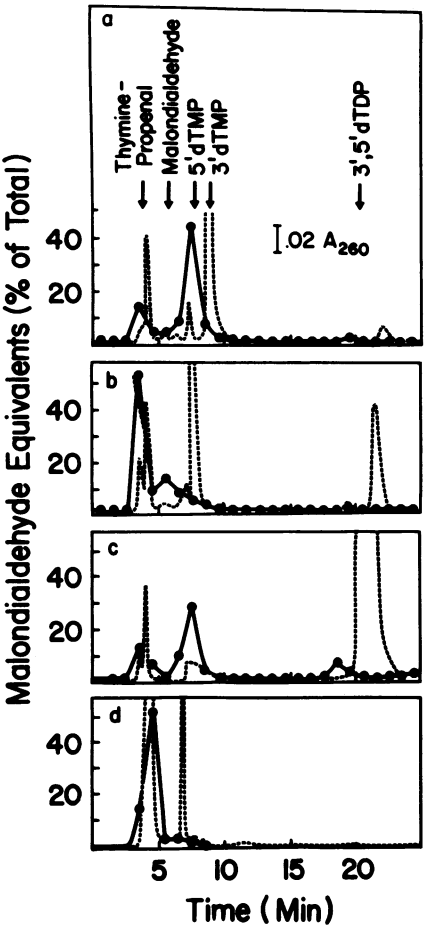

Figure 4. Anion-Exchange Chromatography of Gamma-Irradiated Thymidine Nucleotides. Samples (0.2mM) of a) 3'dTMP (5 umoles), b) 5'dTMP (5 umoles) or c) 3'5'dTDP (12.5 umoles) were gamma-irradiated to 50 krads, concentrated to 1 ml under reduced pressure and resolved by anion exchange HPLC (mobile phase 75 mm KH_2PO_4 (pH 4.5) 0-20 min, linear gradient to 1M KH_2PO_4 (pH 4.5) from 20 min to 30 min) with monitoring at 260 nm. Fractions were collected at 1.0 min intervals and 0.2 ml aliquots assayed for thiobarbituric acid-reactivity. Sample (d) was identical to sample (a) except that the irradiated 3'dTMP was treated with phosphatase (0.40 U, 5h) prior to chromatography. Arrows indicate the elution times for the compounds thymine-propenal, malondialdehyde, 5'dTMP, 3'dTMP and 3',5'dTMP. Symbols as for figure 3.

TBA-reactive material in solutions of thymidine or 3'dTMP suggested to us that TBA-reactive material produced by irradiation of nucleotides might be phosphorylated. To test this hypothesis, solutions of 3'dTMP, 5'dTMP and 3'5'dTDP were irradiated, concentrated under reduced pressure and resolved by anion exchange chromatography (Fig. 4). In each chromatogram some TBA-reactive material appeared in the void volume. Void volume material was further analyzed by silica gel HPLC and found to be largely (59%) thymine-propenal and lesser amounts (up to 40%) malondialdehyde. However, authentic malondialdehyde does not elute in the void volume on anion exchange HPLC and it is likely that it is produced by hydrolysis of thymine-propenal during handling prior to silica gel HPLC. Thus, the TBA-reactive material in

the void volume on anion exchange HPLC is predominantly and could be exclusively thymine-propenal. The TBA-reactive material from irradiated 5'dTMP eluted predominantly in the void volume. In contrast, the major component of TBA-reactive material in solutions of irradiated 3'dTMP was retained (Compound II) and eluted at 7 min, slightly before 3'dTMP. Irradiation of 3'5'dTDP produced TBA-reactive material that eluted with compound II and an additional peak of TBA-reactive material which eluted at 18 min (compound III), immediately before 3'5'dTDP. The amount of compound III varied from 10-40% of the total TBA-reactive material in different preparations of irradiated 3',5'-dTDP. Samples of compound II or compound III retained their characteristic elution times on a second chromatography under the same HPLC conditions. The elution times of compound II and compound III are characteristic of a nucleotide monophosphate and nucleotide diphosphate respectively.

To test the hypothesis that the retained TBA-reactive fractions contain phosphate moieties, solutions of either irradiated 3'dTMP (Fig. 4, panel d) or 3'5'dTDP (data not shown) were treated with calf alkaline phosphatase prior to anion exchange chromatography. Samples treated with phosphatase contain TBA-reactive material only in the void volume, none is retained on anion exchange chromatography. We conclude from these results that solutions of irradiated 3'dTMP or 3'5'dTDP contain a TBA-reactive material with a phosphate monoester moiety.

The presence of compound III only in solutions of irradiated 3'5'dTDP, not in solutions of irradiated 3'dTMP or 5'dTMP, and the elution time of compound III near 3'5'dTDP, suggested that compound III might contain two phosphate groups. If so, then partial dephosphorylation of compound III might produce Compound II, a TBA-reactive compound containing a single phosphate group. To test this possibility, a solution of 3'5'dTDP was irradiated and resolved on anion exchange HPLC. Late eluting fractions containing TBA-reactive material (compound III) were pooled and chromatographed on a second HPLC system after incubation without (Fig. 5a) or with (Fig. 5b) phosphatase. In the absence of phosphatase treatment, compound III elutes at the position of a diphosphate nucleotide with a small amount of TBA-reactive material appearing in the void volume. The phosphatase-treated sample contains a reduced amount of compound III, increased TBA-reactivity in the void volume and TBA-reactive material corresponding to compound II. These data are consistent with conversion of compound III to compound II by removal of a phosphate group.

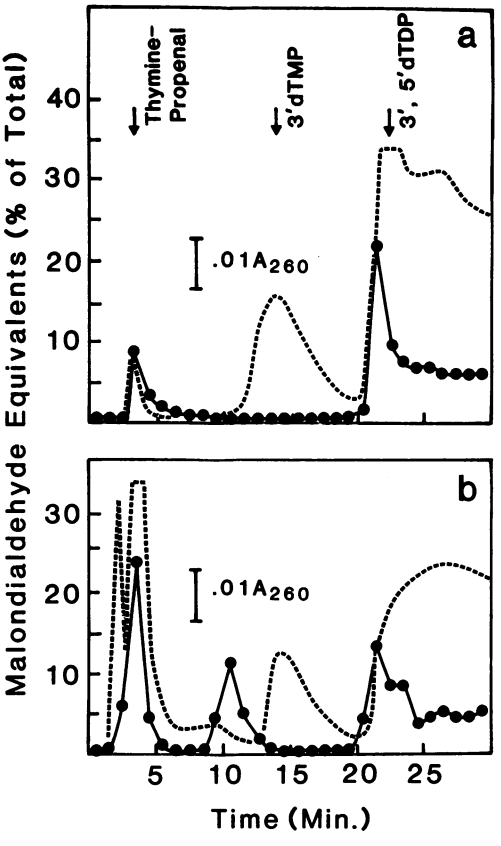

Figure 5. Conversion of Compound III to Compound II by Partial Dephosphorylation. A solution of 3'5'dTDP (0.2 mM) was gamma-irradiated, concentrated and resolved by anion-exchange chromatography as described in Figure 4. The fractions (18-20 min) containing compound III, the late-eluting TBA-reactive material (30 nmoles malondialdehyde equivalents) were pooled and divided into two samples. Samples were incubated without (a) or with (b) phosphatase (0.8 U, 30 min) as described in Methods. Samples were then resolved by a second anion exchange HPLC system (mobile phase 0-15 min, 0.05 M ammonium formate, pH 3.6, at 30 min a step to 1 M ammonium formate, pH 3.6). Elution time for compound II on this system is 10 min. Symbols as for figure 3.

As noted above, complete phosphatase digestion of either compound II or compound III produces TBA-reactive material that is not retained by anion-exchange chromatography. To determine the product of such phosphatase treatment, 3'5'dTDP was irradiated and resolved by anion-exchange chromatography. Fractions containing compound II were pooled. This TBA-reactive material (26.8 nmoles malondialdehyde equivalents) was treated with phosphatase, extracted into acetonitrile and analyzed by silica gel HPLC. Of the malondialdehyde equivalents recovered (17.4 nmoles), 66% was thymine-propenal (compound I), and 34% malondialdehyde. Samples treated in an identical fashion with omission of phosphatase contained no (< 0.5 nmoles) thymine-propenal or malondialdehyde. The identity of the product of dephosphorylation of Compound II as thymine-propenal was further confirmed by comigration with authentic thymine-propenal on silica gel TLC (System II). As noted above, hydrolysis of thymine-propenal to malondialdehyde during handling

may lead to an underestimate of the amount of thymine-propenal on subsequent silca HPLC. In addition, phosphatase treatment of compound II was performed at pH 8.0, and thymine-propenal is slowly hydrolyzed at this pH. Therefore, the amount of thymine-propenal recovered following dephosphorylation of compound II should be considered a minimum estimate. However, we can not eliminate the possibility that dephosphorylation of compound II also produces small amounts of authentic malondialdehyde.

The TBA-Reactive Products of Gamma-Irradiation of DNA. The nature of the TBA-reactive material in solutions of irradiated DNA has also been analyzed by silica gel TLC. Solutions of gamma-irradiated DNA contain thymine-propenal and three other TBA-reactive compounds (Figure 1) with the mobilities of adenine-, guanine- and cytosine-propenals (8). The possibility that a small amount of malondialdehyde (< 5% of the total TBA-reactive material) is present has not been eliminated as malondialdehyde does not completely resolve from cytosine-propenal in this chromatography system.

Most (80%) of the TBA-reactive material following gamma-irradiation of DNA remains at the origin on silica gel chromatography and is therefore neither base-propenal nor malondialdehyde. Furthermore, in agreement with the report by Kapp and Smith (4) we find that the predominant TBA-reactive material in solution of irradiated DNA precipitates (60-80%) with DNA in ethanol. Both malondialdehyde and thymine-propenal are soluble in ethanol in either the presence or absence of carrier DNA (data not shown). To further investigate the nature of the association between TBA-reactive material and DNA, a DNA sample was gamma-irradiated (50 krads) and the DNA precipitated with ethanol. The resuspended DNA was incubated in the presence or absence of Micrococcal nuclease. While both DNA and TBA-reactive material remained ethanol-precipitable in the absence of nuclease, the DNA and TBA-reactive material are both rendered ethanol-soluble by Micrococcal nuclease (Figure 6). This indicates that the TBA-reactive material in solutions of irradiated DNA is ethanol-insoluble because of its linkage to DNA.

DISCUSSION

These studies demonstrate that the TBA-reactive materials produced by gamma-irradiation of DNA, thymidine nucleotides and thymidine are not malondialdehyde but rather compounds which react with TBA to form the same chromophore as that produced by reaction of malondialdehyde with TBA. The malondialdehyde "precursor" produced by irradiation of thymidine has been identified as thymine-propenal. Thymine-propenal has not been described in

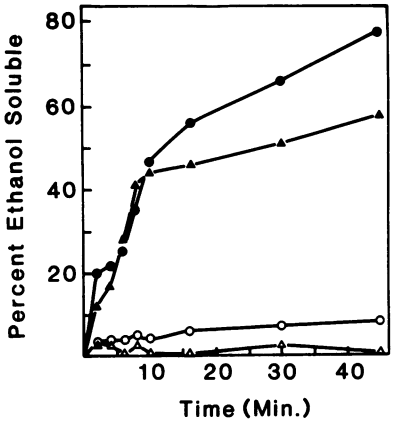

Figure 6. Deoxyribonuclease Sensitivity of TBA-Reactive Material in Irradiated DNA. A solution of calf thymus DNA (10 mM as nucleotide) in 10 mM NaCl was irradiated to 50 krad, ethanol-precipitated and resuspended as described in Methods. Aliquots of 3 umole DNA containing 1.5 nmol MDA equivalents were incubated for the indicated time, with (●,▲) or without (○,△) Micrococcal nuclease as described in Methods. At various times the reaction mixtures were ethanol-precipitated and supernatants were assayed for absorbance at 260 nm (●,○) and for TBA-reactive material (▲,△) as described in Methods.

previous studies of gamma-irradiation of thymidine, although it is produced in amounts equivalent to amounts of individual isomers of thymidine glycol (10). Failure to detect this compound in previous studies may be due to the instability of thymine-propenal at both extremes of pH, conditions which we have avoided during the identification and purification of this compound and also because yields of this compound decline at higher radiation doses.

We propose that in the presence of oxygen, gamma-irradiation of thymidine produces TBA-reactive material by the sequence of reactions shown in Figure 7 and previously described by Giloni and coworkers (8) for bleomycin induced cleavage of DNA. This sequence is consistent with the observed requirement for a deoxyribose moiety in the target molecule and inhibition by free radical scavengers. It is also consistent with the observation by Schulte-Frohlinde and Bothe (11) that gamma-irradiation of thymidine produces equivalent amounts of glycolic acid and "malondialdehyde". As noted above, this reaction sequence is essentially the same as that proposed previously by Giloni et al. (8) and Wu et al. (12) for formation of base-propenal from DNA by the antineoplastic antibiotic bleomycin. In this sequence, in the presence of molecular oxygen, initial abstraction of a hydrogen atom from C-4′ by hydroxyl

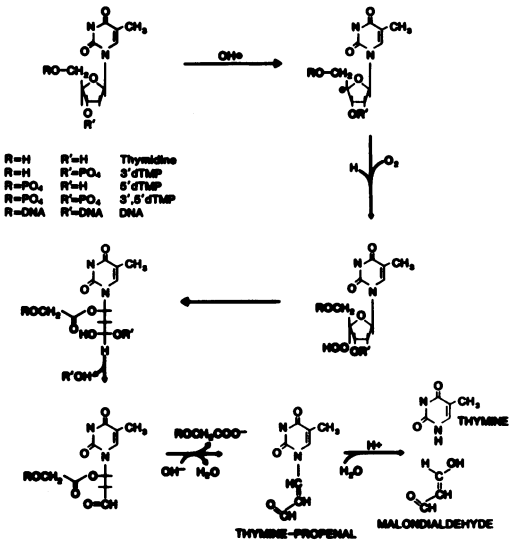

Figure 7. Proposed Mechanism for Formation of TBA-Reactivity in Thymidine, Thymidine Nucleotides or DNA in the Presence of Oxygen.

radical leads to peroxidation at the same site. Rearrangement of the peroxy group then leads to cleavage of the C-3'-C-4' bond. Elimination of water at the hemiacetal produces an aldehyde at C-3'. Abstraction of hydrogen from C-2' releases glycolic acid and thymine-propenal (compound I). Acid can catalyze hydrolysis of thymine-propenal at the C-1'-N-1 bond and produce thymine and malondialdehyde.

The proposed oxygen-dependent mechanism for the formation of glycolate and thymine-propenal does not explain the formation of TBA-reactive products by anoxic irradiation of model compounds lacking 5' phosphoryl linkage (thymidine and 3'dTMP). We have been unable to identify the TBA-reactive material produced during the anoxic irradiation of thymidine or 3'-dTMP, and it is possible, therefore, that the TBA-reactive compounds generated from anoxic solutions differ from those of aerobic solutions. Although the nature of TBA-reactive products in anoxic solutions of thymidine and 3'-dTMP may be of further interest, the existence of such compounds demonstrates the limitations of using thymindine or 3'-dTMP as model compounds for the study of gamma irradiation of DNA. Neither DNA nor 3',5'dTDP yield such compounds upon anoxic irradiation. While these studies have established that the TBA-reactive material produced by gamma-irradiation of thymidine is thymine-propenal, we can not yet assign a definitive structure to the major

thiobarbituric acid-reactive product of gamma-irradiation of thymidine nucleotides. We have been unable to obtain this material in sufficient quantities and purity for complete characterization. The TBA-reactivity in solutions of irradiated 3'dTMP was destroyed by drying or on reverse phase chromatography. However, sufficient quantities were obtained for analysis on anion exchange HPLC chromatography. This analysis reveals that the TBA-reactive material derived from aerobically irradiated 3'dTMP behaves as a monophosphate by the criteria of its retention time and its phosphatase sensitivity. The TBA-reactive material derived from 3',5'dTDP is more complex. Up to 40% has been recovered on anion exchange chromatography as material that is retained as a diphosphate. This presumptive diphosphate is converted to a monophosphate and thymine-propenal by phosphatase action.

Because the structure of these phosphate-containing, TBA-reactive compounds produced by gamma irradiation has not yet been determined, the exact mechanism for their formation is also not established. However, sufficient information is available to formulate a hypothesis which should be useful to test in future studies of the formation and structure of these products. We propose that the phosphate containing, TBA-reactive compounds observed following irradiation of thymidine nucleotides represent intermediates or rearrangement products of the same reaction sequence shown in Figure 7. According to this hypothesis, gamma-irradiation of DNA, deoxynucleotides or deoxynucleosides and bleomycin-treatment of DNA, all produce TBA-reactive material by the reaction sequence shown in Figure 7, but this reaction does not always proceed to completion in nucleotides or polynucleotides in the absence of bleomycin.

There are several similarities between bleomycin-treatment of DNA and gamma-irradiation of DNA that make our postulate of a common reaction sequence plausible. Both release base-propenals containing C'1-C'3 of the deoxyribose and produce 3'phosphoglycolate termini which contain the C'4 and C'5 portions of the deoxyribose (8,12-14). Both require molecular oxygen for the production of TBA-reactive material. However, bleomycin-treatment and gamma-irradiation of DNA also differ in several respects. Production of TBA-reactive material is a quantitatively minor product of gamma-irradiation of DNA (1) while it is the predominant product of bleomycin-treatment of DNA in the presence of molecular oxygen (9). In addition, essentially all of the TBA-reactive material produced in the presence of oxygen during bleomycin-treatment of DNA is released from the polynucleotide as base-propenals (8), while the predominant TBA-reactive material in solutions

of gamma-irradiated DNA remains attached to the polynucleotide. TBA-reactive material that remains attached to the DNA might represent either an early step in the same reaction sequence by which bleomycin produces base-propenals or alternatively might represent a second, independent reaction sequence. However, our observations that production of TBA-reactive material in irradiated DNA or 3'5'dTDP requires oxygen and that phosphatase treatment of irradiated 3'5'dTDP produces thymine-propenal both suggest that TBA-reactive material is produced by the same reaction sequence during either bleomycin-treatment or irradiation of DNA and deoxynucleotides. The extent to which this reaction sequence proceeds largely to completion in the presence of bleomycin may be due to the ability of bleomycin to catalyze both an initial and later step in this sequence, while gamma-rays (or other hydroxyl radical producers) can only initiate this sequence. The essentially enzymatic nature of the bleomycin peptide makes such postulated catalysis by bleomycin plausible.

This report demonstrates that base-propenals are produced by gamma-irradiation of DNA as was previously reported by other investigators for bleomycin treatment of DNA (8). Such formation of base-propenals and precursors to base-propenals in DNA may be responsible for at least a portion of the lethal and mutagenic effects of gamma-irradiation. It has been recently reported (15,16) that base-propenals are highly cytotoxic when added to cell cultures. Base-propenals are reactive with nucleophiles and may exert their cytotoxicity by forming adducts with DNA. If formation of DNA adducts is the mechanism for the cytotoxicity of base-propenals, then formation of base-propenals or precursors to base-propenals within the DNA helix might be substantially more toxic than the same amount of base-propenal added exogenously to cells.

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