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DITHIOTHREITOL-INDUCED OXIDATIVE DAMAGE TO THYMINE AND DNA IN SOLUTION

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SUMMARY: Studies on dithiothreitol-induced oxidative damage to thymine and DNA in solution are reported. The major thymine products, cis- and trans-5,6-dihydroxy-5,6-dihydrothymine (thymine glycols), are produced rapidly in 37°C neutral solutions of 10mM thymine and 10mM dithiothreitol. Iron-EDTA enhances while the iron chelator, diethylenetriaminepentaacetic acid, inhibits the reaction. In experiments using ^{3}H-TdR-labeled Escherichia coli DNA, DNA damage was measured as increased ethanol-soluble radioactivity after treatment of the DNA with 5mM dithiothreitol at 45°C. The findings are important with respect to current research interest in thiol radioprotection and thiol-plus-heat toxicity. © 1987 Academic Press, Inc.

The occurrence of thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) in DNA in vitro or in vivo is generally considered to be evidence of oxidative damage to DNA (1,2). Oxidative DNA damage is implicated as an etiologic factor in a number of human diseases including cancer (3,4). The objective of the present communication is to demonstrate that significant yields of thymine glycol and DNA damage are generated from the oxidation of dithiothreitol in thymine or DNA solutions at physiological pH.

More than a century has elapsed since thiol oxidation was noted by E. Baumann (5); yet, it was not until 1957 that active oxygen species (free radicals) were postulated as intermediaries in iron-catalyzed thiol oxidation reactions (6). Superoxide free radicals ($O_2^{\cdot-}$) from thiol oxidation were measured by Misra (7), and Rowley and Halliwell (8) in 1981 verified the presence of ·OH in thiol oxidation reactions. The fact that iron is a potent catalyst of thiol oxidation has been verified by a number of authors (5,6,8-10); the Fe-complex-oxygen recycling reactions are now often referred to as "iron-catalyzed Haber-Weiss reactions" (10).

Abbreviations: DTT , dithiothreitol; DHD = trans-4,5-hydroxy-1,2-dithiane, DTPA , diethylenetriaminepentaacetic acid; KP , mono- and dibasic potassium phosphate buffer, 10 mM; pH 7.

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Biological buffers often contain the essential ingredients for efficient thiol oxidation (11). Once oxidation mechanisms are initiated, it seems likely that the ·OH produced can affect cells or model systems treated with thiol containing solutions. This is the first report the author is aware of that demonstrates formation of thymine glycol, a major oxidation product of ·OH and thymine reactions, from the oxidation of dithiothreitol in buffered aqueous solutions. Hydroxyl-radical mechanisms have been suggested as a mechanism of cellular toxicity in heated, thiol-treated cells (12,13); however, the present report is the first direct evidence that this mechanism is feasible under physiological conditions.

MATERIALS AND METHODS

Chemical preparations. DTT and DHD (oxidized dithiothreitol) were obtained from Sigma Chemical Company (St.Louis, MO) and were used without additional purification. In the preparation of DTT and DHD solutions, the dry powdered chemical was added to solution immediately before the start of a given experiment. Alternatively, the buffers were first degassed with N_2 and chilled (0-2°C) before addition of the compounds. In either case, excessive DTT oxidation was prevented before the start of an experiment.

Potassium and sodium phosphates were Fisher reagent quality or better. The iron content of concentrated stock solutions was measured by colorimetry using bathophenanthroline. In all cases, iron concentrations were well below the specified maximum impurity levels on the bottle labels.

DTPA was obtained from Aldrich Chemical Company and was used without further purification. Disodium EDTA (Na_2EDTA) and iron EDTA (FeEDTA) were Sigma chemicals and also used without further purification.

The thymine solutions were prepared from thymine (Eastman Chemicals) and high purity distilled water which was prepared by distillation, charcoal filtration, deionization and a second distillation. Preparation of water used for HPLC and all buffers included sterilization by filtration through 0.2 μm pore size membrane filters.

Preparation of ^{3}H-DNA. Stock cultures of Escherichia coli K12 cells, strain AB1157 (ATCC 29055) were grown on M9CA supplemented with glucose (0.2%) and thiamine·HCl (1 mg/l). The cells were labelled using ^{3}H-[methyl]thymidine, 60 Ci/mmol, at a concentration of 10 μCi/ml in the overnight broth. Cells were harvested at titers of 1-2 x 10^9 cells-ml^{-1} and chromosomal DNA was isolated using standard biochemical techniques including buffered phenol and chloroform-isoamyl alcohol extractions (13). The yield of DNA was determined using 1 O.D. (260 nm) = 50 μg/ml and a purity check for a 260 nm/280nm absorbance ratio = 1.8. DNA that did not meet the purity criteria were re-dialized in TNE buffer at 0-5°C. The final preparation of ^{3}H-DNA in potassium phosphate buffer (10mM, pH 7.0) was performed by first ethanol-precipitating the DNA from TNE buffer. The precipitated DNA was dried thoroughly before redissolving it in KP buffer using 37°C and gentle agitation.

Preparation of 2'-deoxynucleosides for HPLC analysis. DNA that had been treated with various combinations of DTT and modifiers was precipitated in ethanol (which also quenches any further ·OH reactions), dried and then redissolved in Tris buffer, pH 8.0. The duplex molecule was digested to 5'-deoxynucleotides using DNase I followed by digestion to 2'-deoxynucleosides using snake venom phosphodiesterase and bacterial alkaline phosphatase. The resulting digest was filtered through a 10-kda centrifuge ultrafilter (Centricon 10, Amicon Co.). The filtrate, < 10 kda, was injected directly into the HPLC. Fractions were collected and the ^{3}H content determined using liquid scintillation counting.

HPLC methods. HPLC methods have been reported elsewhere (15) and in essence were performed using a simple water flow gradient for the measurement of the thymine-DTT mixtures, and a flow-mobile phase gradient for the analysis of 2'-deoxynucleoside mixtures. The buffered mobile phase consisted of $(NH_4)H_2PO_4$ (pH 4.4,0.15 M) over acetonitrile.

UV detection was at 210 nm which is a useful wavelength for a variety of ring-damaged thymine oxidation products such as thymine glycol. Quantitative analysis of peak areas was performed using a Spectra Physics 4270 Integrator. Peak integrals were calibrated using co-chromatography with authentic standards.

Thymine and DNA oxidation experiments. Stocks of thymine and "modifiers", such as FeEDTA and DTPA were prepared fresh in 10 mM KP buffer before the experiments. The thymine mixtures were placed in foil-wrapped tubes in a covered waterbath set at the appropriate temperature. Immediately before the experiment, a fresh stock of DTT was prepared and rapidly added to the appropriate mixtures. At prescribed times after mixing, aliquots of the mixtures were removed and quickly chilled to icebath temperatures. The samples were stored frozen (-20°C) until analysis by HPLC.

Radiolabeled DNA experiments were performed in an analogous manner with the addition of sampling 10 or 20 µl aliquots for liquid scintillation counting at various stages of processing. Thiol-DNA reaction were stopped by addition of two volumes of ice-cold (-15°C) ethanol which also served to precipitate DNA. Counts in the ethanol-soluble fraction or in the enzyme DNA digest were expressed as a percent of the initial to radioactivity in the sample. All experiments were repeated 2-4 times and the data were averaged before plotting or calculations were made.

RESULTS AND DISCUSSION

A representative chromatogram of thymine oxidation products induced by DTT oxidation is shown in Figure 1. Among the thymine products found were trans- and cis-thymine glycol, 6-hydroxy-5,6-dihydrothymine and several other products in relatively smaller yields. The focus of the present study are the glycols; thus, the non-glycol products will not be discussed here.

The experimental solutions containing mixtures of ±DTT, ±EDTA, and ±DTPA were all chromatographed on the same gradient system which enabled rapid comparison of the results. Cis- and trans- thymine glycol peaks were trailing peaks on the DTPA and EDTA peaks; however, they were easily quantitated using electronic integration.

The quantitative yield of thymine glycol in solutions containing DTT is shown in Figure 2. In terms of radiation-equivalent yields, approximately 120 Gy-equivalents of TG is produced with 8 hrs in a heated (37°C) solution containing DTT and thymine. As predicted by Lambeth (11) and Rowley and Halliwell (8), FeEDTA greatly enhanced and DTPA greatly inhibited this reaction. Na_2EDTA also enhanced thymine glycol formation, indicating that some residual Fe contamination was present in the Na_2EDTA-thiol-thymine mixtures (not shown). Thus, iron-like activity, in the solutions in which FeEDTA and Na_2EDTA were not present, probably indicates the presence of iron contaminants.

Chromatograms illustrating the effect of FeEDTA ± DTT mixtures on DNA in solution are shown in Figure 3, and the quantitative results are shown in

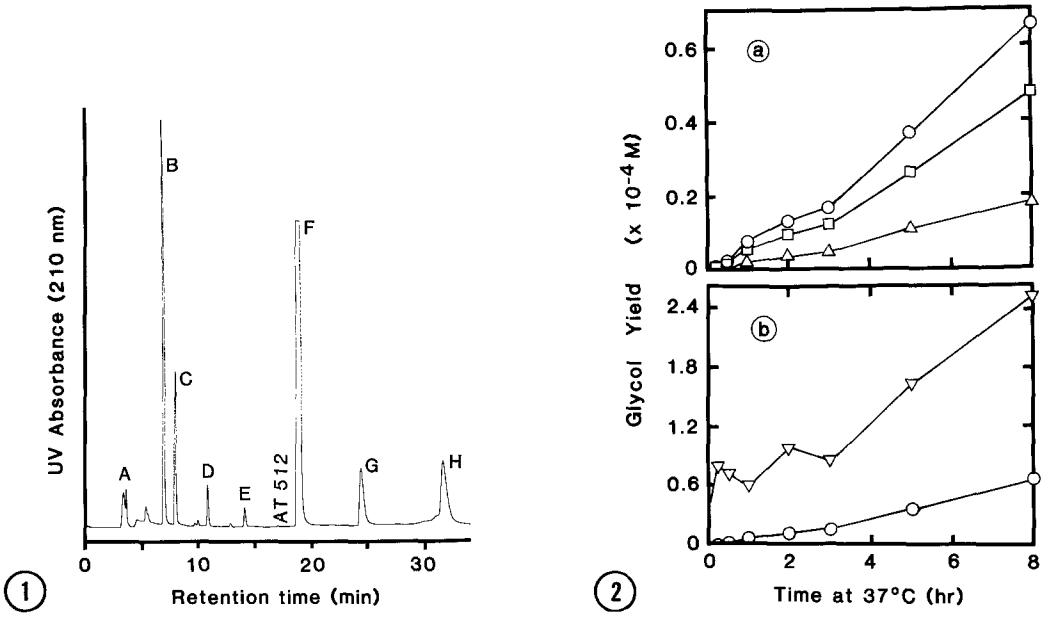
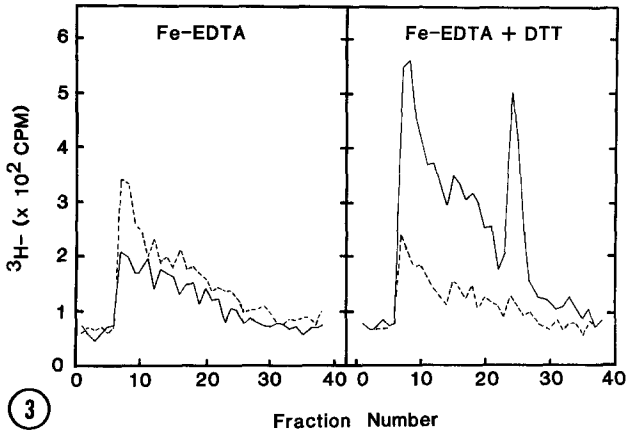


Figure 1. HPLC chromatogram of a thymine and DTT mixture. The mixture was chromatographed 8 hr after mixing thymine (10 mM) and DTT (10 mM) at 37°C and pH 7. The sensitivity is 0.05 AUFS and the attenuation was increased 16-fold at 17.5 min order to show the thymine peak (F) and the DTT peaks (Peak G = reduced form and Peak H = oxidized DTT). The thymine peak has been "clipped" because it exceeded the 0.05 AU output sensitivity. Peak identities: A = buffer and unknown; B = trans-thymine glycol; C = cis-thymine glycol; D = (tentatively) 6-hydroxy-5,6-dihydrothymine; E = unidentified.

Figure 2. Thymine glycol yields in thymine DTT mixtures at 37°C. Panel (a): Total glycol yield = (o); trans-thymine glycol yield = (□); and cis-thymine glycol = (△). In panel (b), the total thymine glycol yield for mixtures of thymine + DTT with (▽) and without (O) added FeEDTA (0.25 mM) are shown. In these experiments, thymine and DTT were initially present at 10 mM each in potassium phosphate buffer (10 mM, pH 7). Samples containing added DTPA or "no DTT" did not have any detectable thymine glycols. The data shown are the average of two separate experiments.

Figure 4. These results show that DNA base release, measured as released, ethanol-soluble radioactivity from ^{3}H-thymine labeled DNA, occurred somewhat in DTT-containing solutions and occurred rapidly in DTT + FeEDTA solutions. The latter finding was anticipated in view of the recent application of FeEDTA for hydroxyl radical "footprinting" of DNA (16,17). On a relative yield basis, the DNA double-stranded state is protective of thymine (i.e., only 10 Gy-equivalents [net] formed in 4 hrs). The lowered yield of base damage in the duplex molecular compared to free bases in solution is in line with results by Ward et al, for ionizing radiation induced base damage (18). In the present case, it is also likely that some of the ·OH radicals are consumed in strand-break reactions. The evidence for this is in the chromatograms (Figure 3) that show a large radioactivity gradient in early retention times. This gradient could contain "n-mers" of polynucleotides up to the 10-kDa cut-off of the ultrafilters used to purify the product. Verification of the



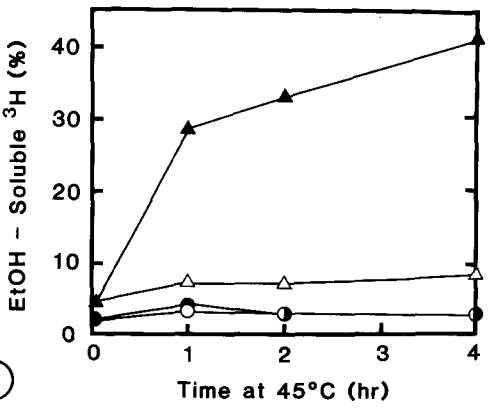


Figure 3. Representative radiochromatograms of "base release" products from ^{3}H-DNA + FeEDTA +/- DTT. Chromatography samples were taken from the ethanol-soluble portions of the reaction mixtures at 2 (----) and 240 (——) min after heating at 45°C. The left panel shows 0.25 mM FeEDTA + (approx.) 100 µg/ml DNA. The right panel shows the effect of added 5 mM DTT on base release. The peak at 24 min is thymidine, and the glycols would normally appear at retention times of 8-11 min in this system.

Figure 4. Quantitative base release measurements on DTT - ^{3}H-DNA mixtures. The symbols are the averages of two independent experiments showing: DNA + DTT (5 mM) + FeEDTA (0.25 mM) = (▲); DNA + FeEDTA = (△); DNA + DTT = (●); and DNA alone = (○). These results indicate that 30% of the DNA ^{3}H-TdR present is released in 1 hr of heating DNA-DTT-FeEDTA mixtures. The radioactivity accounts for base release (damaged and undamaged TdR's) and strand breaks producing oligonucleotides < 10 kDa in size.

oligonucleotide content would entail additional experiments using (e.g.) gel electrophoresis.

CONCLUSION

The present report shows that the oxidation of a thiol in a neutral buffer can cause significant damage in DNA and its constituents. These results indicate the necessity of complete controls (i.e., monitoring thiol oxidation) in studies on the hyperthermic enhancement of thiol toxicity and thiol radioprotection. Current studies in our laboratory will attempt to elucidate the magnitude of these effects in cells using Escherichia coli cells of known DNA repair capacity. Preliminary results show that some thiol radioprotection might result from induction of DNA repair pathways such as "SOS" repair in E. coli K12.

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