in vivo RADIATION-INDUCED THYMINE RESIDUE RELEASE FROM *E. coli* DNA

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SUMMARY: Cells of *E. coli* C thy⁻321 are examined for thymine residue release from DNA following gamma-irradiation from 5 to 15 krad. Experimental conditions are designed to inhibit enzyme activity that might promote base residue release. Enzyme action is restricted in order to assess the physicochemical action of radiation on cellular DNA, and to this end irradiation is done under O_2, N_2, and N_2O saturating conditions. Both thymine and thymidine release from bacterial DNA are detected and quantitated, and three oxygen effects are noted in comparing yields of these products. No difference in effect is observed between N_2 and N_2O gassing conditions, suggesting that the hydroxyl radical has little effect on thymine or thymidine release from irradiated DNA *in vivo*.

INTRODUCTION

The elucidation of specific effects of ionizing radiation on DNA *in vivo* has so far proved relatively difficult beyond characterizing strand breaks. An indirect reductive assay has been reported as a method for measuring a class of thymine damage products in DNA of, for example, irradiated *M. radiodurans* (1). In contrast, reports on damage in DNA irradiated in aqueous solution include chromophore destruction (2); base (3-5) and nucleoside (4,5) release; sugar damage (5,6); and peroxide formation (7). Similar kinds of damage might be expected to occur in DNA irradiated *in vivo*. Work is in progress in this laboratory to directly identify and quantitate DNA base damage produced in *E. coli* cells *in vivo*. In approaching this work it was deemed necessary to inhibit enzymatic repair systems, and to determine whether or not some fraction of the DNA base damage products are released from, rather than retained in, the DNA in the absence of repair. The results of these studies indicate that significant quantities of apparently undamaged thymine, thymidine (and perhaps some thymidylic acid) are released from the cellular DNA of gamma-irradiated *E. coli* at biologically significant doses and under environmental conditions which are generally thought to inhibit enzymatic action. Previous reports indicate that thymine alone (8) or thymidine alone (9), or both thymine and phosphorylated thymidine (10) are released from irradiated *E. coli*. However, these measurements were all made after post-irradiation incubation.

MATERIALS AND METHODS

Preirradiation:

A 0.1 ml innoculum of E. coli C thy⁻321 (provided by Dr. Roger Hewitt, M. D. Anderson Hospital, Houston, Texas) was drawn from a stationary phase culture and transferred to 100 ml of TCG medium (11) made up to 3.4 x 10^{-5} M thymine, 1% glucose, 10^{-4} M $CaCl_2$, and 1 mCi of [^{3}H]-6-thymine (Nuclear Dynamics) added. Cells were grown to late log phase in shaker culture at 37°C, and harvested by centrifugation. The difference in radioactivity content of the medium before and after growth was used to calculate percent thymine uptake (70 to 80%), specific labelling of cell DNA (1 [^{3}H] thymine for every 98 thymine bases incorporated), and DNA sample weight for direct action G-value determinations. Cells were resuspended in 200 ml of fresh medium without [^{3}H]thymine and incubated for an additional 70 min (1.3 generation times) in order to clear radioactive thymine from the metabolic pool and obtain a fully log phase cell population. Cells were washed twice with pH 6.8 phosphate buffer (1/30 M monobasic:1/30 M dibasic potassium phosphate), and resuspended in 10 ml of buffer. This suspension was then divided into 5 ml volumes which served thereafter as control and irradiated samples.

Irradiation:

Cell suspension samples were placed in an ice bath, equilibrated with either N_2, O_2, or N_2O bubbling (80 ml/min), and irradiated in a ^{60}Co gamma cell with continued gassing throughout the irradiation interval. Dose rate was 947 rad/min determined by ferrous sulfate dosimetry.

Postirradiation:

Cell suspensions were maintained at ice bath temperature for 45 min following irradiation in order to allow for equilibrium diffusion of chemical species from the cells. Cells were then centrifuged at 0°C, and washed twice with 25 ml of ice bath-prechilled phosphate buffer made 0.1 M in disodium EDTA. No cell lysis was apparent during this washing procedure. Samples were resuspended in 5 ml of prechilled 0.15 M sodium chloride:0.015 M sodium citrate solution at pH 7.0 (SSC) made 0.1 M in disodium EDTA, and this suspension made 1% in sodium lauryl sulfate (SLS). These suspensions remained in an ice bath for 3 hr, after which time they were a viscous jelled mass (cell lysis assumed complete at this point). Protease (Sigma, type VI; 0.005 g/ml) was added, and digestion carried out with shaking at 37°C for 12 hrs, followed by dialysis (Union Carbide viscous cellulose tubing, 24 Å pore size) against 3 changes of 9 volumes of water. The dialysant (defined here as the solution external to the dialysis tubing) was collected, lyophilized, and redissolved in 2.5 ml of water.

These dialysant samples, estimated to contain better than 95% of the small molecular weight products originating from the bacterial main-chain DNA, were purified for subsequent analysis by precipitating a) SLS with addition of excess 1 M $BaCl_2$; b) excess barium by addition of 2 M Na_2SO_4; and c) EDTA, large polypeptides, and DNA fragments by addition of 5 volumes of absolute ethanol. Samples were evaporated to dryness, resolubilized in 1 ml of water, and desalted by gel filtration using a 65 x 0.9 cm column of Sephadex G-10 with elution at 0.26 ml/min using pH 4.0, 0.1 M ammonium acetate solution. The elution profile, consisting of 2.6 ml fractions, was standardized with thymidylic acid (TMP), thymidine (TdR) and thymine (T) (found to elute in this order with little overlap). Radioactive fractions from the experimental elution profiles were found corresponding to T, TdR, and TMP (this latter with extensive overlap of void volume/oligonucleotide activity), and were collected, lyophilized, and concentrated to a 1 ml volume. Thymine and thymidine fractions were further subjected to a 3-fold ether extraction using 5 ml of ether per extraction. The ether phases were pooled, 2 ml of absolute ethanol added, evaporated to dryness, and these samples resolubilized with 1 ml of water.

At various steps above in treating the dialysant, radioactivity was measured from 20 µl samples in 5 ml of Bray's solution using a Packard Tri-carb Model 2425 liquid scintillation counter. The final fractions (both ether and aqueous phases) were assayed by one-dimensional thin layer chromatography (TLC) using $(20\ cm)^2$ sheets of PEI-cellulose (Machery-Nagle polygram Cel300) developed with n-butanol:methanol:water:ammonia (60:20:20:1). Developed chromatograms were scanned by a two-dimensional windowless radiochromatogram counter (Varian-Berthold Model LB242K). Radioactivity was graphically recorded, and the areas under the resultant curves electronically integrated and compared with a standard of known activity to obtain quantitative estimates of product yields. From the radioscans, the R_f values for T, TdR, and TMP were found to be 0.65, 0.69, and 0.02, respectively. The separation of T from TdR in this chromatographic system, though small, is very constant, and TdR without exception runs slightly ahead of T.

RESULTS

The radioactivity in cpm from the dialysant samples of the E. coli protease digest following 5, 10, and 15 krad of gamma irradiation under different gassing conditions are shown in Figure 1. All values are averages from re-

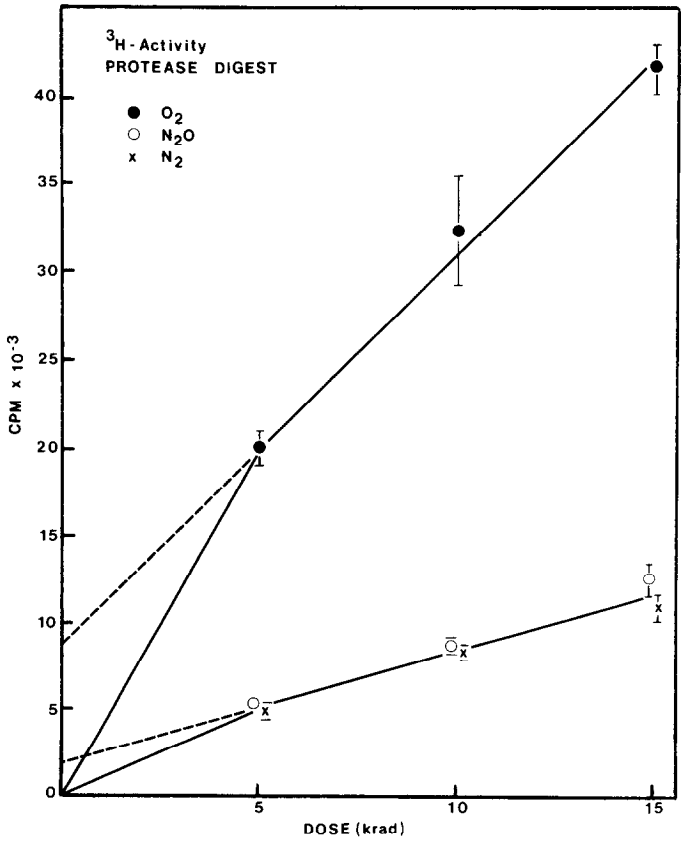

Figure 1: [^{3}H]-activity detected by liquid scintillation of protease digest dialysant samples following irradiation under O_2, N_2, or N_2O saturation.

peated experiments: points without error bars are derived from two experiments; points with error bars (standard error of the mean) are derived from three to five experiments. This is the case for both figures. These values are control subtracted. The control values are fairly constant for all experiments, and amount to 5% or less of the irradiated values.

When the samples of protease digest dialysant are further purified through gel filtration and the thymine and thymidine fractions collected, the radioactivity is found to be that shown in Figure 2. These samples have been concentrated 2.5 fold relative to those of Figure 1, so some loss of activity is evident during the purification and fractionation procedures. Despite these losses of sample material during purification, the relative profiles remain comparable with those of Figure 1, in that: a) more labilized product persists from irradiated oxic than from anoxic cell suspensions; b) little difference is observed between N_2 and N_2O saturation conditions; and c) a more rapid appearance of product occurs below 5 krad than above. However, Figure 2 also reveals the significant observation that the T/TdR ratio from cells irra-

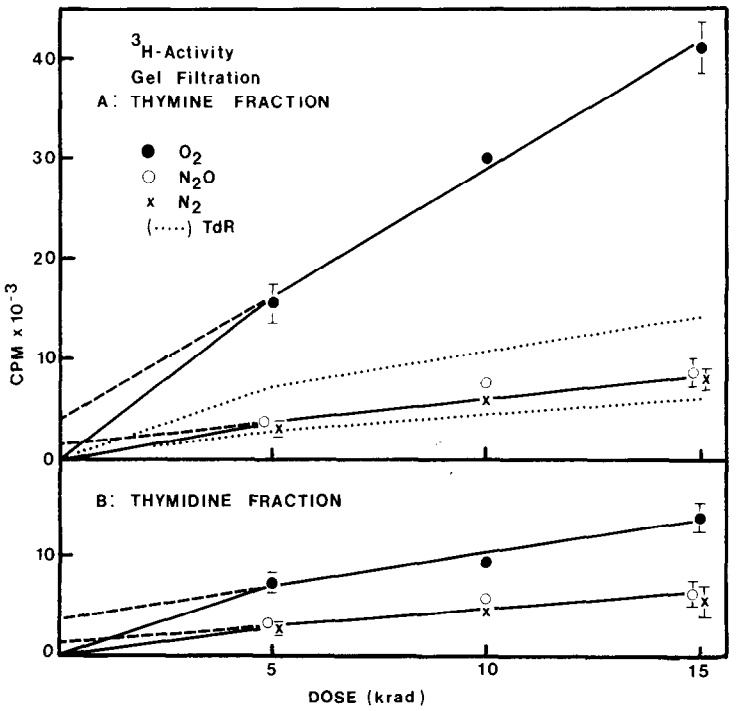

Figure 2: [^{3}H]-activity detected by liquid scintillation of T and TdR fractions following gel filtration of protease digest dialysant samples. Samples concentrated 2.5 fold relative to samples in Figure 1.

diated in O_2 (3.12 at 15 krad) is much greater than for cells irradiated in N_2 or N_2O (1.33 at 15 krad).

The TdR and T fractions from Figure 2 are subjected to ether extraction and developed by TLC on PEI-cellulose. The absolute yields of T and TdR from TLC are found to correspond to 81-94% of the initial sample radioactivity, depending on the experiment. These yields, derived from the 15 krad experiments, are listed in the Table as G-values for thymine and thymidine release, and as percent of initial [^{3}H]uptake. G-values are calculated on the basis of both direct and indirect action.

Confidence in the identity and quantity of TMP is not secure at this time due to oligonucleotide contamination. Upper-limit determinations for TMP release indicate that yields are much below those of T and TdR. Further work is needed before TMP yields and identity can be determined conclusively.

YIELDS OF THYMINE RESIDUE RELEASE[a]

Gassing Condition	% of Initial T Uptake		G-value Indirect Action[b]		G-value Direct Action[c]	
	TdR	T	TdR	T	TdR	T
O_2	0.167	0.468	0.029	0.082	87	243
N_2	0.066	0.083	0.012	0.015	34	42
N_2O	0.064	0.085	0.011	0.015	33	43

a) calculated from 15 krad experiments
b) G=number release/100 eV/g cell suspension
c) G=number release/100 eV/g cell DNA

DISCUSSION

The appearance of thymine alone (8), thymidine alone (9), or both thymine and thymidylic acid derivative(s) (10) (but not both T and TdR) from irradiated E. coli following enzymatic DNA degradation during post-irradiation incubation has been documented. Results reported here on labilized thymine residues, however, are the first derived from irradiated cells under conditions designed to restrict enzyme action. If the observed release of T and TdR in these experiments is attributable to the physicochemical action of radiation, then it must be assumed that enzymatic DNA degradation is defeated by ice bath temperature and phosphate followed by 0.1 M EDTA, SSC, and SLS cell lysis/protein denaturation. In this work, doses of gamma radiation are also kept within a range of biological relevance. For example, 5 krads results in 60% sur-

vival of E. coli C in air saturated and ca. 90% survival in N_2 saturated suspensions.

Various gassing conditions are studied on the assumption that products observed might be related to the physicochemical actions of radiation. If this is the case, radical reactions in the presence of O_2 might be expected to yield different results compared with N_2 gassing. Irradiation under N_2O saturated conditions is also studied to determine whether an increased hydroxyl radical yield in this case might also provide differences in product type and/or quantity. That there is no difference, at least to the extent of T and TdR release, between the N_2 and N_2O experiments is surprising.

Three oxygen effects are obvious from the results. First, more thymine and second, more thymidine, is seen in the presence of O_2 than in its absence. Third, the relative amount of T compared to TdR is greater for oxic experiments than for anoxic ones. These results differ from those found previously from post-irradiation incubation experiments in which the degree of E. coli DNA degradation was reported to be the same for cells irradiated in O_2 or N_2. The presence of O_2 after, but not during, irradiation is important for this degradation (8).

If the hypothesis that base residue release occurs as a result of the physicochemical effect of radiation (no enzyme action) is accepted, then a comparison with results reported for in vitro DNA irradiation is appropriate. Both base and nucleoside release are reported from such work with DNA aqueous solutions. Pertinent observations from oxygenated solutions include: base release (3-5, and John Ward, personal communication), with G(total base release) = 0.06 (5), G(T release) = 0.1 (Ward, personal communication) and 0.05 (4); nucleoside release with G(total release) = 0.1 (5); and relatively rapid thymine (3) and base residue (5) release at low doses (50 krad being the lowest dose reported). In no case is nucleotide release observed from irradiated DNA solutions.

The above observations from irradiated DNA in vitro qualitatively compare with the results from experiments we report here. Additionally, the G-values for base and nucleoside release, which would have been computed on the basis of indirect action, agree reasonably with the indirect action G-values listed in the Table. The in vitro experiments described above which report G-values for base and nucleoside release utilize DNA concentrations in a range from 0.5 mg to 5 mg DNA/ml. Our concentration of 0.67 mg DNA/ml (DNA residing in the cell, of course) falls within this range. It is perhaps only coincidence that approximately equivalent in vivo and in vitro DNA sample content results in approximately equivalent indirect action G-values. However, the microsphere environment surrounding DNA in the bacterial cell is not known, and appre-

ciable reactions with radicals generated from water radiolysis may be possible. Work involving B. megaterium spores suggests that radicals from water radiolysis are important for a component of radiation damage in cells (12,13).

The generally acceptable G-value calculation for intracellular events is made assuming direct action. Our values (see Table) are too high to be strictly related to direct events of radiolysis. The reasonable upper limit for G-values on the basis of direct action would be ca. 4 (14). By way of comparison, in vivo DNA single strand breaks generally are found with G $\sim$ 1.5 (15,16).

If the hypothesis of direct action is accepted, then the most reasonable explanation for our excessive direct action G-values is to assume that enzyme action is not totally blocked by the restrictive conditions placed on these experiments. Base residue damage in DNA in vivo could establish lesions which are recognized by an enzyme system as starting points for degradation. Radiolytically excised residues may form a class of lesions biologically distinct from sites produced by enzymatic degradation. If enzymatic processes provide the thymine residue release we observe, then the nature of the enzyme is of great interest since it must operate in high phosphate concentrations at ice bath temperature and/or in the presence of 0.1 M EDTA, SSC, and 1% SLS. It must be much more efficient in acting on lesions produced in the presence of O_2 than in N_2 or N_2O as evidenced by the pronounced O_2 effect described above. We are not aware of any degradative enzyme reported to date that has been shown to utilize a DNA substrate under these conditions.

Endonuclease II, an enzyme known to act on alkylated and apurinic DNA, conceivably might be a candidate here. It is promoted by Mg^{2+}, but does not have an absolute metal requirement. However, it cannot tolerate 10^{-3} M EDTA or 0.05% sodium dodecyl sulfate (17). Most other endonucleases appear to be metal requirers (although activation by divalent cations not chelated by EDTA is not generally examined). Further, the number of endonuclease sensitive sites in DNA irradiated in O_2 is reported to be less than when O_2 is absent (18); a trend that would seem to be the reverse of yields observed in our case.

Irradiated DNA could also serve as substrate for exonuclease action. In this regard, the recBC nuclease has been shown to be involved in the degradation of DNA in E. coli during post-irradiation incubation (10). However, requirements of this enzyme have been reported to include Mg^{2+} (19). In addition, Cerutti (20) has reported in vitro experiments which implicate the 5'$\rightarrow$3' exonucleolytic activity associated with DNA Polymerase I in the excision of a class of damaged thymine products and undamaged thymine in irradiated phage DNA.

It is conceivable that an enzyme exists in E. coli that detects thymine nucleotide damage, and specifically hydrolyzes the N-glycosyl (or sugar-phosphate) bond to result in the base residue products reported here. Very recently, Lindahl has described such a specific enzyme (21). This is an N-glycosidase from E. coli, and is specific for cleaving the base uracil from deoxyribose following cytosine deamination in double stranded DNA. Such an enzyme, in itself or representative of a new class of enzymes, could perhaps recognize thymine nucleotide damage in irradiated DNA and act specifically in its excision. The uracyl N-glycosidase is independent of the presence of Mg^{2+} and phosphate, but is largely inhibited by 0.1 M NaCl.

Considering (a) that the radiochemical assay techniques described here are sensitive in detecting product yields of G (direct action) less than one at biologically relevant doses, and (b) that the E. coli repair enzyme systems lend themselves well to genetic and chemical inhibition, further experiments now in progress in this laboratory on in vivo DNA damage should provide more information on the physicochemical effects of radiation in cells and the subsequent processes of enzymatic DNA degradation and repair. It is hoped that such results may also aid in clarifying both the state of DNA in cells and the validity of in vitro model systems.

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