Free Rad. Res. Comms., Vol. 18, No. 1, pp. 17–28
Reprints available directly from the publisher
Photocopying permitted by license only

^{32}P-POSTLABELING ASSAY FOR FREE RADICAL-INDUCED DNA DAMAGE: THE FORMAMIDO REMNANT OF THYMINE

ALEXANDER E. MACCUBBIN,* EDWIN E. BUDZINSKI, HELEN PATRZYC, MARIANNE S. EVANS, NOREEN ERSING* and HAROLD C. BOX

*Biophysics Department and *Department of Experimental Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263, USA*

(Received May 19th, 1992; in final form September 13th, 1992)

A prominent lesion in DNA exposed to oxidative free radicals results from the degradation of thymine leaving a formamido remnant. A ^{32}P-postlabeling assay has been developed for the detection of the formamido lesion. The assay is based on the circumstance that the lesion prevents hydrolysis by nuclease P1 of the phosphoester bond 3′ to the damaged nucleoside. Thus, a nuclease P1 plus acid phosphatase digest of DNA generates mostly nucleosides whereas the formamido lesion is rendered as a modified dinucleoside monophosphate. Dinucleoside monophosphates, but not nucleosides, are apt substrates for ^{32}P-postlabeling by polynucleotide kinase. The assay was applied to calf thymus DNA X-irradiated in oxygenated solution. The formamido lesion could be detected down to a dose of a few Gy.

KEY WORDS: DNA damage, ^{32}P-postlabeling, formamido lesion.

INTRODUCTION

Free radicals are generated *in vivo* by a variety of mechanisms including oxidative stress, redox cycling and ionizing radiation. The most dire biological consequences of free radical generation are likely to follow from damage produced in DNA. Over the past several years the DNA lesion most frequently assayed as an indicator of free radical-induced DNA damage has been the 8-hydroxyguanine lesion. The recent prominence of the 8-hydroxyguanine in the literature of DNA damage is due in part to the development of a sensitive and facile assay for the detection of this lesion.[1] Relevant also is the fact that the 8-hydroxyguanine lesion is one of the most abundant base damages produced in DNA by free radicals. When a tetramer containing the four DNA nucleosides is irradiated in oxygenated solution the damaged oligomer obtained in second largest yield contains the 8-hydroxyguanine lesion. The damaged oligomer obtained in largest yield is one in which thymine has been degraded to a formamido remnant (i.e., -HN-HCO).[2] The formamido lesion was originally characterized by Teoule *et al.*[3,4] If oxygen is substantially purged from the solution by a flow of nitrous oxide before and during irradiation, the effect is to convert solvated electrons into additional hydroxyl radicals. Under these conditions, the 8-hydroxyguanine modification of d(TpApCpG) remains prominent, but the formamido modification is much reduced.

Formation of the 8-hydroxyguanine lesion has been associated with oxidative stress and implicated in carcinogenesis.[5] Malins and Haimanot[6] found this lesion significantly enhanced in breast cancer. It has also been associated with the aging process.[7] Similar associations have not been made with the formamido lesion. It is not clear whether the dependence on oxygen for its formation diminishes the importance of the formamido lesion in biological systems. This seems unlikely because *in vitro* even a very low oxygen pressure results in formamido production. It may be that the formamido lesion has been largely ignored simply because it is difficult to monitor. In any case the latter difficulty must be overcome before the former question can be addressed.

In order to detect DNA damage that may occur at low levels in biological systems sensitive techniques must be used. Among the sensitive methods that have been developed to detect and quantify lesions in DNA, the ^{32}P-postlabeling assay is one of the most sensitive.[8-10] This method has been widely applied for detection of DNA adducts formed by bulky, aromatic carcinogens.[11] However, the application of this technique to small, hydrophilic lesions, having thin layer chromatographic characteristics similar to the normal nucleotides, has been limited. We have been developing modifications of the ^{32}P-postlabeling assay that allow detection of radiation damaged nucleotides. The assay (Fig. 1) provides selectivity for the lesion using nuclease P1 digestion[12,13] and enrichment of the lesion using HPLC.[14] The formamido lesion, as well as certain other radiation-induced lesions, block the digestion of DNA by nuclease P1.[15,16] Thus, when irradiated DNA is digested with nuclease P1 and acid phosphatase, the digest contains modified dinucleoside monophosphates in the form of d(N*pN) where N* is a damaged nucleoside such as the formamido remnant. Dinucleoside monophosphates, but not nucleosides, are phosphorylated in the ^{32}P-postlabeling step of the assay. Initial enrichment of the lesion is achieved by HPLC separation of dinucleoside monophosphate containing the formamido lesion from other digestion products. The enriched product is ^{32}P-labeled and separated from other labeled material by thin layer chromatography (TLC). The inclusion of a UV-visible marker for the phosphorylated product allows the radioactively labeled product to be tracked during the TLC separation.

In this report we describe the development of the ^{32}P-postlabeling assay for the formamido lesion. Results are presented demonstrating the detection of the formamido lesion in calf thymus DNA exposed to X-irradiation.

MATERIALS AND METHODS

Sample Preparation

Calf thymus DNA (Cooper Biochemical) was X-irradiated in aqueous solutions to the prescribed dose. The solution, in a gold plated cup, was agitated before and during irradiation by a stream of oxygen gas. The irradiation of 1 mg of DNA in 10 ml of phosphate buffered water, pH 7.0, was carried out at room temperature. A model dimer, d(TpA), was irradiated under the same conditions to generate the dimer d(T^FpA) where T^F stands for the formamido remnant of thymidine. This product was purified and characterized as previously described[17] and used as a standard for optimizing postlabeling conditions. It was also used to synthesize the carrier employed in TLC measurements described below.

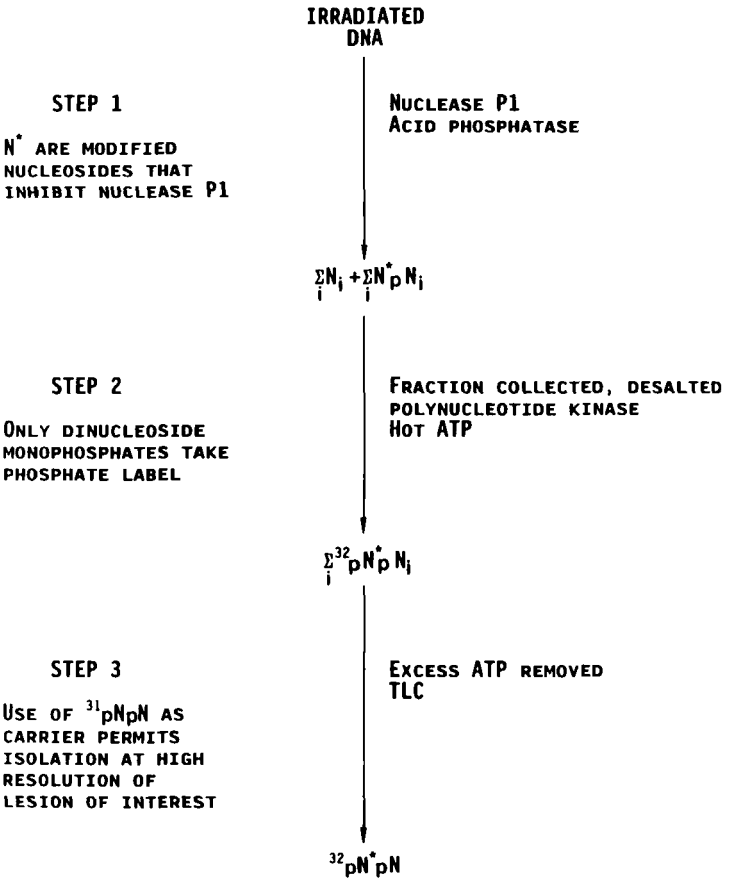


Figure 1 Proposed scheme for assaying nuclease P1-resistant lesions. DNA is digested with a combination of nuclease P1 and acid phosphatase generating nucleosides, N_i, and modified dinucleoside monophosphates, N^*pN_i, where *N** contains the modified base. The digest is separated and fraction(s) collected, desalted and radiolabeled by polynucleotide kinase-mediated transfer of ^{32}P from $\gamma^{32}P$-ATP. After removing the excess ATP using a solid phase extraction column; the radiolabeled products are separated by 2-dimensional TLC in the presence of UV-detectable carrier, namely nonradioactive phosphorylated dinucleoside monophosphate.

DNA Digests

Digestions of DNA were carried out using 1 ml solutions containing 100 μg of calf thymus DNA. A nuclease P1 treatment consisted of the addition of 1.44 μl of 30 mM $ZnCl_2$, 240 μl of 0.25 M sodium acetate (pH 5.0) and 0.48 μg of the enzyme (Boehringer Mannheim). The mixture was incubated at 37°C for 3 hrs. For dephosphorylation, 3.3 μl of a solution containing 100 mU per μl of prostatic acid phosphatase (Sigma) was added. Reactions were terminated by adding 23.3 μl of 0.1 M 2[N-cyclohexylamino]-ethane sulfonic acid (CHES) buffer, pH 9.5. Each enzyme treatment was repeated three times.

HPLC

The DNA digest was chromatographed by injection onto an Ultremex 3μ C18 RP column (Phenomenex) and elution at 0.2 ml/min for 30 minutes with 0.1 M ammonium acetate, followed by a 60 min 0–10% acetonitrile gradient in 0.1 M ammonium acetate at a flow rate of 2 ml/min, followed by isocratic elution at 2 ml/min with 10% acetonitrile in 0.1 M ammonium acetate. A fraction containing the d(T^FpA) dinucleoside monophosphate was collected, desalted and postlabeled. Desalting was accomplished by a second HPLC run using 1 mM ammonium acetate with a 30 min. 0–50% methanol gradient.

^{32}P-Postlabeling

Dimers and DNA digests were ^{32}P-labeled as described previously[18] with the following modifications. The amount of polynucleotide kinase was increased to 10 units per reaction and unlabeled ATP was added to a final concentration of 60 μM. These conditions were found to increase the labeling efficiency of the modified dimer (see Results). The postlabeling reaction was terminated by the addition of apyrase and incubation at 37°C for 30 min.[8] The postlabeled sample was diluted to 100 μl with 50 mM triethylamine acetate, pH 7.0 (TEAA), and applied to a preequilibrated C18 solid phase extraction column (Sep-Pak, Waters, Milford, MA). Inorganic phosphate from unused ATP, destroyed by apyrase, was washed from the column with 50 mM TEAA; subsequently, the postlabeled dimers were eluted with 50% acetonitrile contained in 50 mM TEAA. The eluted sample was dried in a vacuum concentrator (SpeedVac, Savant Corp.). The dried sample was redissolved in 20 mM Bicine buffer, pH 9.5, spiked with a non-radioactive marker, and spotted on a preequilibrated PEI-cellulose TLC plate.

Two-dimensional TLC of Postlabeled Products

The sample was spotted on a 20 × 20 cm TLC plate that had been prewashed in methanol and water followed by equilibration with 0.225 M ammonium formate, pH 3.5. The plate was then developed from bottom to top with 2.25 M ammonium formate, pH 3.5, followed by rinsing in distilled water and methanol and drying. The plate was turned 90° and chromatographed in the second dimension in 1.0 M LiCl. The development again was to the top of the plate. After this chromatographic step, the plate was dried, wrapped in Saran Wrap and autoradiographed.

Evaluation of Adduct Level

Based on autoradiography and detection of the UV marker, the modified dimer spot was cut from the TLC plate and counted by liquid scintillation spectrometry. After liquid scintillation counting the TLC spot was washed in methanol and the radioactivity and UV marker were eluted from the plate with 1 M NaCl.[18] An aliquot of this sample was run on HPLC to confirm that the radioactivity was incorporated in the modified dimer.[18] Based on the specific activity of the ATP,[19] the amount of DNA in the original digest and the radioactivity in the spot, the adduct level was calculated by the formula: adduct in fmol/μg DNA = [(radioactivity in spot)/(specific activity)]/μg digested.

RESULTS

From studies on oligomers irradiated in oxygenated solution it was concluded that the formamido lesion was the principal oxidative damage and that this lesion inhibited hydrolysis by nuclease P1.[2,15,16] These conclusions have now been shown to apply to DNA itself. Fig. 2 shows the HPLC elution profile of a nuclease P1 (plus acid phosphatase) digest of calf thymus DNA after irradiation (400 Gy) in oxygenated solution. The 8OH-dG nucleoside and d(T^FpA) dinucleoside monophosphate products are identified. In calf thymus DNA the yield of d(T^FpA) is less than that of 8OH-dG, but it must be remembered that the lesion is produced in other guises also, namely d(T^FpG), d(T^FpC) and d(T^FpT). We pursued only the d(T^FpA) form of the lesion in our attempt to design an assay which would allow detection of the lesion at lower doses of radiation. In addition to damaged dinucleoside monophosphates, the elution profile in Fig. 2 contains peaks due to residual amounts of normal dinucleoside monophosphates, most notably d(TpT). Only the dinucleoside monophosphates d(CpC) and d(CpG) elute in the vicinity of d(T^FpA),[20] but are hydrolyzed below the levels observable in Fig. 2. The possible effect of small residuals of d(CpC) and d(CpG) on the proposed assay is considered below.

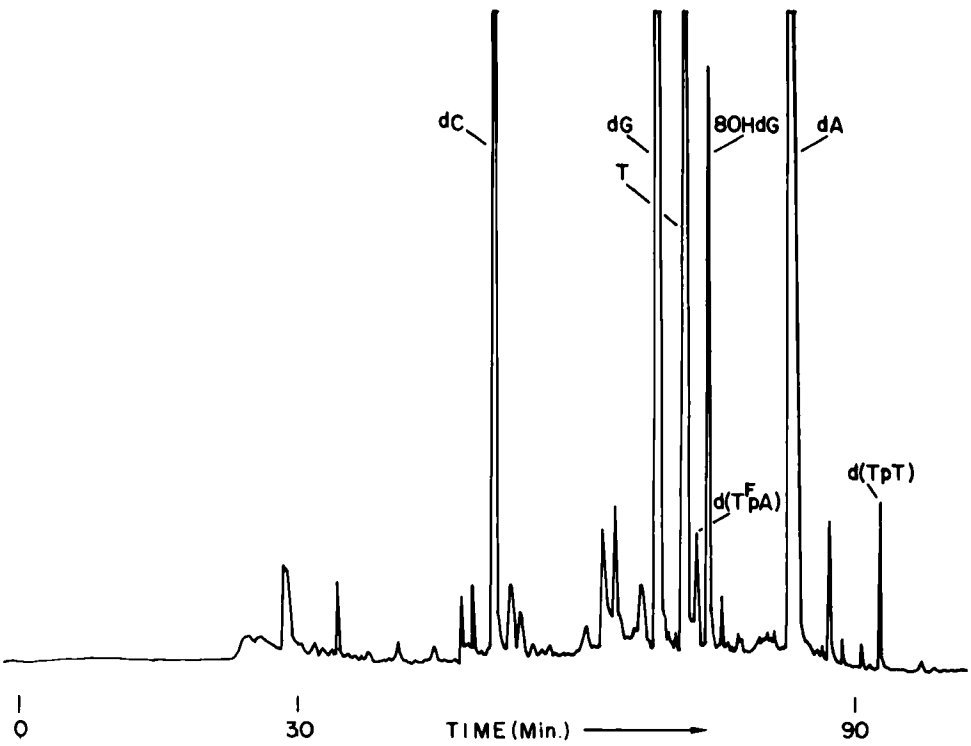


Figure 2 The HPLC elution profile of DNA X-irradiated (400 Gy) in oxygenated solution and subsequent digestion by nuclease P1 and acid phosphatase. The formamido modification of d(TpA) elutes after thymidine and before 8-hydroxyguanosine.

Although normal dinucleoside monophosphates are suitable substrates for postlabeling by polynucleotide kinase, the question arises whether a modified dinucleoside monophosphate such as d(T^FpA) is a suitable substrate. Preliminary results indicated that under our standard condtions using 50 μCi γ^{32}P-ATP and 3 units of polynucleotide kinase[18] less than 10% of the d(T^FpA) was phosphorylated. Increasing the concentration of ATP increased the labeling of d(T^FpA) as shown in Fig. 3. With 10 units of polynucleotide kinase, concentrations of ATP greater than 30 μM gave optimal labeling. Thus, we used 10 units of polynucleotide kinase and 60 μM ATP in all subsequent labeling experiments.

Phosphorylation of d(T^FpA), using polynucleotide kinase and cold ATP, provided carrier for spiking the radioactivity labeled produced dispersed on the TLC plate in step 3 of the assay (Fig. 1). The ultraviolet absorption of the cold carrier serves to locate the product of interest on the TLC plate and as a marker for fraction collection in HPLC separations.

Based on the observed resistance of the d(T^FpA) dimer to digestion by nuclease P1 and acid phosphatase, we initially explored the possibility of using a recently reported modification of the ^{32}P-postlabeling assay[13] for detection of the d(T^FpA) dimer. Irradiated DNA was digested and the products were postlabeled and separated by TLC. The resulting map demonstrated the presence of many nuclease P1-resistant products that were postlabeled, some of which overlapped the product of interest. It

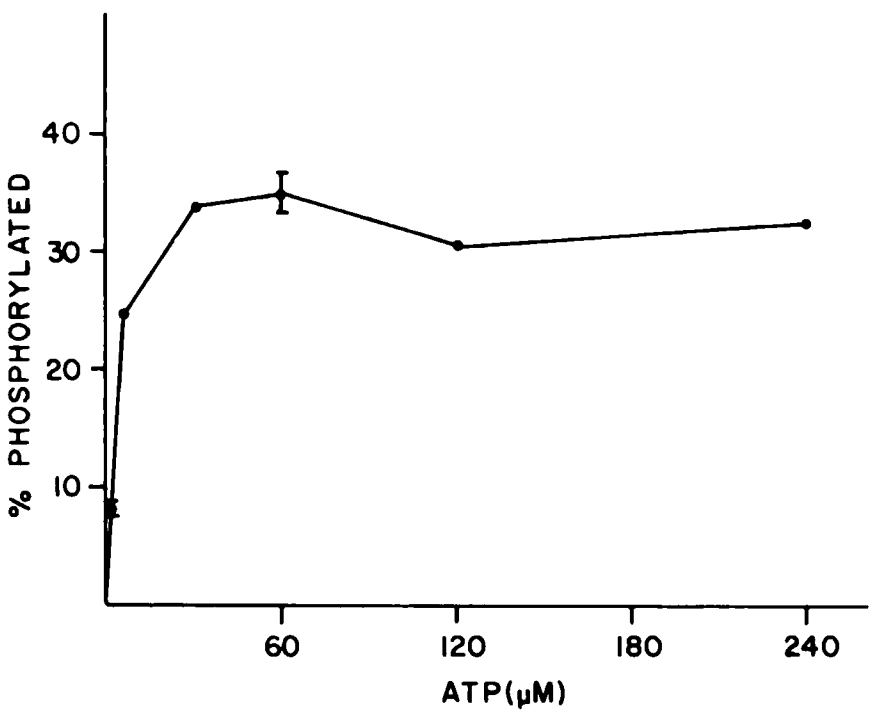


Figure 3 Dependence of phosphorylation reaction on ATP concentration. Aliquots of d(T^FpA) were phosphorylated by polynucleotide kinase in the presence of varying concentrations of ATP. After 45 min at 37°C, the products were separated by HPLC or TLC and the percent phosphorylated calculated from the measured amounts of d(pT^FpA) and d(T^FpA).

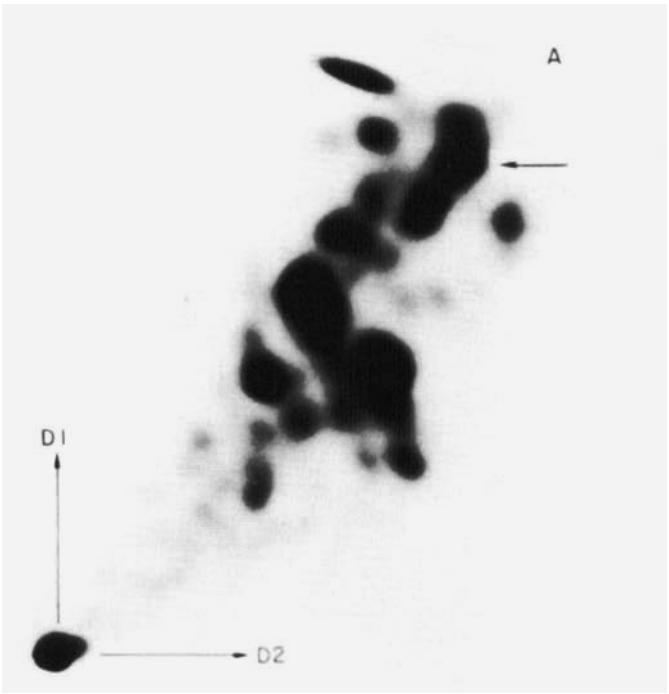
seemed advisable therefore to take advantage of HPLC, prior to postlabeling, to enrich the lesion content of the sample by further reducing undigested normal dinucleoside monophosphates. Calf thymus DNA was X-irradiated in oxygenated solution to various doses. Following digestion by nuclease P1 and acid phosphatase, the interval of the HPLC elution profile containing the d(T^FpA) product (see Fig. 2) was collected. After desalting and labeling, the lesion of interest was resolved by two-dimensional TLC. The location of the product is defined by the carrier which is observable visually on the TLC plate by virtue of its ultraviolet absorption and by autoradiography (Fig. 4). The TLC map obtained after enrichment by HPLC still contains spots other than d(pT^FpA), including for example, a small amount of unmodified d(pCpC). In order to unambiguously determine the amount of d(T^FpA) that was present in the DNA digest, the spot defined by the UV detectable carrier was excised from the TLC plate. The total radioactivity in the spot was measured by liquid scintillation spectrometry and the radioactivity eluted from the TLC piece with 1 M NaCl. In all samples, the elution step released >95% of the radioactivity. An aliquot of the eluted sample was analyzed by HPLC and fractions were collected. The carrier added prior to TLC also served as a marker for the dimer in this HPLC step. As can be seen in Fig. 4b, the bulk of the radioactivity in the TLC spot coelutes with the standard thus confirming that the radiolabeled spot was incorporated in the d(T^FpA) dimer. The moles of d(T^FpA) per microgram of DNA was calculated from the percentage of the total radioactivity contained in the TLC spot after HPLC isolation of the product. The dose response for d(T^FpA) formation in calf thymus DNA is shown in Fig. 5. The results show that the assay is able to detect the formamido lesion with a signal well above background for a dose of 6 Gy.

The data of Fig. 5 reflect a yield of d(T^FpA) of 18 femtomoles per microgram of DNA per Gy. This corresponds to 1.8 d(T^FpA) lesions formed per 10^5 thymine bases per Gy. The G value for this product is 0.0019 micromoles per joule. Assuming all four d(T^FpN) are formed in equal abundance the overall yield of the formamido lesion is 0.0076 micromoles per joule. We are not aware of reports of comparable measurements of the formamido lesion. It is interesting to note, however, that Dizdaroglu[21] obtained a G value of 0.03 micromoles per joule for the 8-hydroxyguanine lesion from calf thymus DNA irradiated in aerated solution. Weinfeld and Soderlind[22] obtained a G value of 0.0022 for thymine glycol from calf thymus DNA irradiated in air-saturated solution. From previous studies on DNA oligomers irradiation in oxygenated solution, the yields of the 8-hydroguanine lesion and of the formamido lesion were expected to be comparable and considerably larger than the yield of glycol.[2,23]

Measurements of oxidative damage in DNA are delimited by background levels. We measured a background level for d(T^FpA) of 11 femtomoles per microgram corresponding to 1.10 lesions per 10^5 thymine. The overall background for formamido lesion is estimated as 4.4 per 10^5 thymine. This level of background may be compared with background levels of 3 per 10^5 G and 22 per 10^5 G reported by Frankel *et al.*[24] and Floyd *et al.*[25] respectively for the 8-hydroxyguanine lesion in DNA.

DISCUSSION

A ^{32}p-postlabeling assay has been described for the detection of a DNA lesion, namely the formamido remnant of thymine.[3,4] The lesion may be an important lesion in the



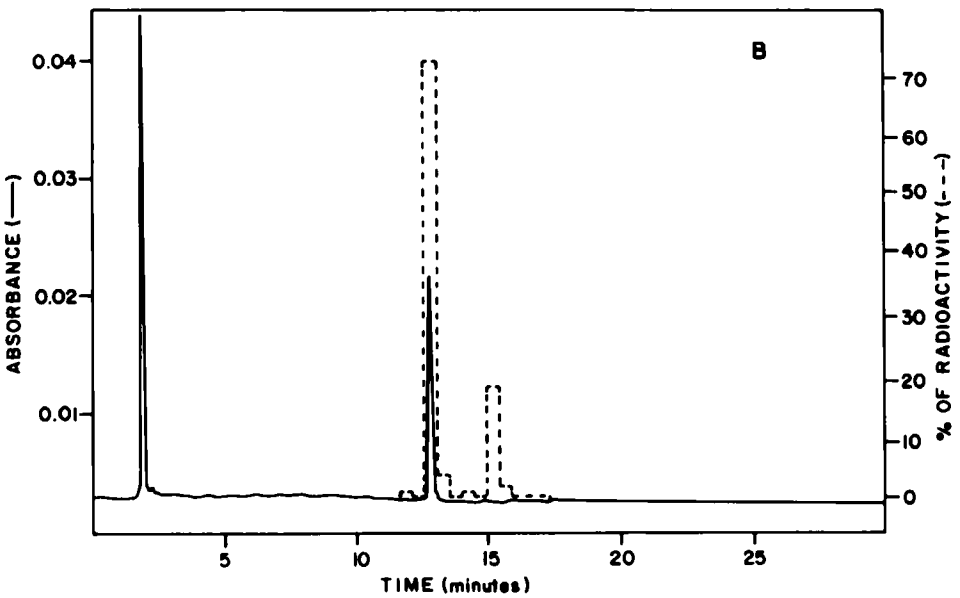


Figure 4 (a) Autoradiogram of TLC of ^{32}p-postlabeling material collected from the region of the HPLC elution profile in Fig. 2 containing d(T^FpA). Carrier in the form of d(pT^FpA) was added to the sample. The location of d(pT^FpA) was determined by autoradiography and UV-absorbance indicated by arrow. (b) Further resolution of d(pT^FpA) from other products was achieved by eluting the spot from the TLC plate with NaCl and separating the eluted products by HPLC. The bulk of the radioactivity coeluted with the UV-marker.

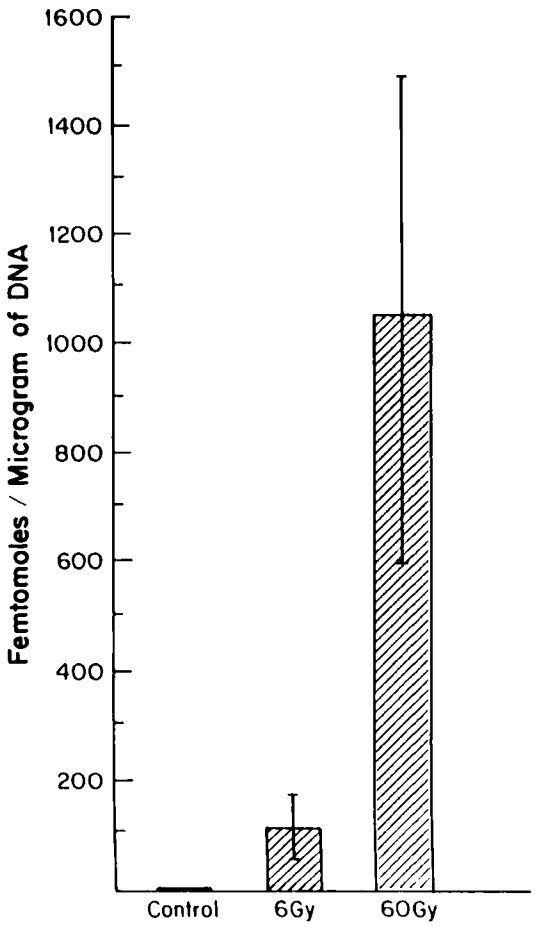


Figure 5 Yield of formamido lesion as a function of dose. Calf thymus DNA was irradiated with X-rays to the indicated dose. The DNA was treated as outlined in Fig. 1 and d(pT^{F}pA) was quantitated by liquid scintillation spectrometry. The values are the mean of duplicated samples at each dose and have been corrected for labeling efficiency and for loses as described in text.

DNA of cells subjected to ionizing radiation or oxidative stress. We find the lesion is prominent when DNA is X-irradiated in oxygenated solution. To our knowledge this is the first report of the use of ^{32}P-postlabeling to detect the formamido lesion. We are hopeful an assay for the formamido lesion will prove especially insightful since this lesion is prominent when DNA is irradiated in the presence of oxygen. The presence of oxygen is also required for the generation of superoxide. Although superoxide *per se* is relatively unreactive, it can generate hydrogen peroxide that ultimately leads to DNA damage. Thus, one may speculate that the generation of superoxide and production of formamido lesion are associated phenomena.

The assay is unusual because the formamido lesion is detected as a modified dimer. The fact that the lesion is detected at the dimer level has at least three distinct advantages. First, dinucleoside monophosphates can be selectively labeled using T4 polynucleotide kinase and radioactive ATP. The bulk of the DNA digest is in the

form of nucleosides which are not acceptable substrates for the T4 kinase and consequently are not phosphorylated. Second, after postlabeling, but prior to TLC (see Fig. 1), it is of considerable advantage to remove radioactivity due to inorganic phosphate or unused ATP from the DNA digest. This is readily accomplished using solid phase extraction columns (e.g., SEP-PAK C18 cartridges). Complete removal can be accomplished because the DNA lesions are manipulated at the dimer level; removal of unwanted material would not be effective if the lesions were manipulated at the monomer level. Third, radioactive postlabeling is an extremely sensitive method for detecting DNA damage. However studies indicate that the TLC assay conventionally used in ^{32}P-postlabeling assays will not distinguish among the many types of lesions introduced into DNA by ionizing radiation or by oxidative stress. The last step in the assay (Fig. 1) permits better discrimination against background through the use of carrier bearing the lesion of interest.[26,27] A subtle advantage attends the use of dinucleoside monophosphates as carriers. Because most radiation-modified pyrimidines have a negligible absorption in the ultraviolet, they are difficult to detect and make poor carriers. In contrast, the carrier in our assay is readily detected because of the residual absorption of the modified nucleoside of the dimer. The lesion is quantitated by measurement of radioactivity cochromatographed with the carrier.

In evaluating the proposed assay several sources of error must be considered. There are five steps in the procedure where loss of product may be a consideration: (1) Formamido in the form of d(T^{F}pA) may escape detection if not all possible product is digested down to the dimer level or if the modified dimer is digested further to the monomer level. Neither possibility seems likely to contribute significant error.[16] (2) Collection of the appropriate fraction of the DNA digest from the HPLC elution profile may result in some loss of product. However, this step was investigated experimentally and found to be ninety percent efficient or better. (3) As a precaution against variable results in the postlabeling step, the samples selected from the HPLC profile were desalted using an alcohol gradient in 1 mM ammonium acetate. As in the previous HPLC step recovery is 90% or greater. (4) The phosphorylation (postlabeling) step was discussed above. As shown in the results section for the conditions used in this assay, the efficiency of phosphorylation is about 30%. (5) The solid phase extraction step following labeling is important because it removes most of the unused radioactivity from the sample, thus reducing background. Over 90% of unincorporated ATP is removed and more than 85% of the labeled dimer is recovered.[28]

The assay depends upon the fact that nuclease P1 does not hydrolyze the phosphoester bond 3′ to a nucleoside bearing the formamido lesion. A comparison of the ability of nuclease P1 to hydrolyze 16 dinucleoside monophosphates showed lysis was effected most efficiently when the 5′ nucleoside was either deoxyadenosine or deoxycytidine. Efficiency decreased with deoxyguanosine and was poorest for thymidine.[20] These results suggest that the availability of appropriate sites on the 5′ nucleoside for hydrogen bonding to the enzyme is an important determinant for substrate binding.[29] These binding sites are eliminated in d(T^{F}pA), perhaps thereby establishing a basis for our assay.

Acknowledgements

This work was supported by grants CA25027, CA46838 and CA44808 from the National Cancer Institute.

References

1. R.A. Floyd, J.J. Watson, P.K. Wong, D.H. Atmiller and R.C. Richard (1986) Hydroxyl free radical adduct of deoxyguanosine: Sensitive detection and mechanisms of formation. *Free Radical Research Communications*, **1**, 163–173.
2. C.R. Paul, J.C. Wallace, J.L. Alderfer and H.C. Box (1988) Radiation chemistry of d(TpApCpG) in oxygenated solution. *International Journal of Radiation Biology* **54**, 403–415.
3. R. Teoule, A. Boncel, C. Bert, J. Cadet and M. Polverelli (1974) Identification of radio products resulting from the breakage of thymine moiety by gamma irradiation of E. coli DNA in an aerated aqueous solution. *Radiation Research* **57**, 46–58.
4. R. Teoule, C. Bert and A. Boncel (1977) Thymine fragment damage retained in the DNA polynucleotide chain after gamma irradiation in aerated solution *Radiation Research* **72**, 190–200.
5. R.A. Floyd (1990) The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis* **11**, 1447–1450.
6. D.C. Malins and R. Haimanot (1991) Major alterations in the nucleotide structure of DNA in cancer of the female breast. *Cancer Research* **51**, 5430–5432.
7. C.G. Frage, M.K. Shigenta, J.-W. Park, P. Degan and B.N. Ames (1990) Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proceedings of the National Academy of Sciences, USA* **87**, 4533–4537.
8. K. Randerath, M.V. Reddy and R.C. Gupta (1981) ^{32}P-labeling test for DNA damage. *Proceedings of the National Academy of Sciences, USA* **78**, 6126–6129.
9. R.C. Gupta, M.V. Reddy and K. Randerath (1982) ^{32}P-postlabeling analysis of nonradioactive aromatic carcinogen-DNA adducts. *Carcinogenesis* **3**, 1081–1092.
10. N.V. Reddy, R.C. Gupta, E. Randerath and K. Randerath (1984) ^{32}P-postlabeling test for covalent DNA binding of chemicals *in vivo*: application to a variety of aromatic carcinogens and methylating agents. *Carcinogenesis* **5**, 231–234.
11. W.P. Watson (1987) Postlabeling for detecting DNA damage. *Mutagenesis* **2**, 319–331.
12. M.V. Reddy and K. Randerath (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts. *Carcinogenesis* **7**, 1543–1551.
13. K. Randerath, E. Randerath, T.F. Danna, K.L. van Golan and K.L. Putman (1989) A new sensitive ^{32}P-postlabeling assay based on the specific enzyme conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5'-monophosphate. *Carcinogenesis* **10**, 1231–1239.
14. B.P. Dunn and R.H.C. San (1988) HPLC enrichment of hydrophobic DNA-carcinogen adducts for enhanced sensitivity of ^{32}P-postlabeling analysis. *Carcinogenesis* **9**, 1055–1060.
15. C.R. Paul, E.E. Budzinski, A.E. Maccubbin, J.C. Wallace and H.C. Box (1990) Characterization of radiation-induced damage in d(TpApCpG). *International Journal of Radiation Biology* **58**, 759–768.
16. A.E. Maccubbin, M.S. Evans, E.E. Budzinski, J.C. Wallace and H.C. Box (1992) Characterization of two radiation-induced lesions from DNA: studies using nuclease P1. *International Journal of Radiation Biology* **61**, 729–736.
17. C.A. Belfi, A.V. Arakali, C.R. Paul and H.C. Box (1986) Radiation chemistry of a dinucleoside monophosphate and its sequence isomer. *Radiation Research* **106**, 17–30.
18. A.E. Maccubbin, L. Caballes, J.M. Riordan, D.H. Huang and H.L. Gurtoo (1991) A cyclophosphamide/DNA phosphoester adduct formed *in vitro* and *in vivo*. *Cancer Research* **51**, 886–892.
19. M.V. Reddy, W.T. Bleicher and G.R. Blackburn (1991) ^{32}P-postlabeling detection of thymine glycols: evaluation of adduct recoveries after enhancement with affinity chromatography, nuclease P1, nuclease S1 and polynucleotide kinase. *Cancer Communications* **3**, 109–117.
20. H.C. Box, E.E. Budzinski, M.S. Evans, J.B. French and A.E. Maccubbin (1992) The differential lysis of phosphoester bonds by nuclease P1. Submitted.
21. M. Dizdaroglu (1985) Formation of an 8-hydroxyguanine moiety in deoxyribonucleic acid on γ-irradiation in aqueous solution. *Biochemistry* **24**, 4476–4481.
22. M. Weinfeld and K.-J.M. Solderlind (1991) ^{32}P-postlabeling detecting of radiation-induced DNA damage: Identification and estimation of thymine glycols and phosphoglycolate termini. *Biochemistry* **30**, 1091–1097.
23. C.R. Paul, C.A. Belfi, A.V. Arakali and H.C. Box (1987) Radiation damage to dinucleoside monophosphates: mediated versus direct damage. *Journal of Radiation Research* **51**, 103–114.
24. K. Frenkel, Z. Zhong, H. Wei, J. Karjoszka, U. Patel, K. Rashid, M. Georgescu and J. Solomon (1991) Quantitative high-performance liquid chromatography analysis on DNA oxidized *in vitro* and *in vivo*. *Analytical Biochemistry* **196**, 126–136.
25. R.A. Floyd, M.S. West, K.L. Eneff, W.E. Hogsett and D.T. Tingley (1988) Hydroxyl free radical mediated formation of 8-hydroxyguanine in isolated DNA. *Archives of Biochemistry and Biophysics* **262**, 266–272.

26. V.L. Wilson, A.K. Basu, J.M. Essigmann, R.A. Smith and C. Harris (1988) O^6-Alkyldeoxyguanosine detection by ^{32}P-postlabeling and nucleotide chromatographic analysis. *Cancer Research* **48**, 2156–2161.
27. P.G. Shields, A.C. Povey, V.L. Wilson and C.C. Harris (1990) Combined high pressure liquid chromatography/^{32}P-postlabeling assay of N^7-methylguanosine. *Cancer Research* **50**, 6580–6584.
28. A.E. Maccubbin, L. Lee, N. Ersing and H.L. Gurtoo. The use of C18-solid phase extraction columns in the ^{32}P-postlabeling assay. In preparation.
29. A. Volbeda, A. Lahn, F. Sakiyama and D. Suck (1991) Crystal structure of *Penicillium citrinum* at 2.9Å resolution. *EMBO Journal* **10**, 1607–1618.

Accepted by Prof. B. Halliwell