Preparation of a Methylated DNA Standard, and Its Stability on Storage

Martin R. Osborne* and David H. Phillips

Molecular Carcinogenesis Section, Institute of Cancer Research, Haddow Laboratories, Cotswold Road, Sutton, Surrey SM2 5NG, U.K.

Received October 28, 1999

Several methods are available for the determination of low levels of O^6-methylguanine in DNA; this base arises after methylation by environmental carcinogens. The reliability of these assays is much improved by the use of a standard. We have prepared such a standard by treating calf thymus DNA with [^{3}H-*methyl*]-*N*-methyl-*N*-nitrosourea. We characterized the methylated bases by hydrolysis of a sample to nucleosides, followed by liquid chromatography and liquid scintillation counting of the tritium content. The level of O^6-methylguanine was 0.6 per one million nucleotides. This base is stable, and its level was unchanged after storage for 5 years at −20 °C. The methylated DNA also contained 7-methylguanine and 3-methyladenine, which are slowly cleaved from the DNA on standing. The half-life for loss of 7-methylguanine at neutral pH was estimated to be 70 h at 39 °C, 460 h at 22 °C, 3800 h at 10 °C, and about 4 years at −20 °C.

Introduction

With the advent of several sensitive methods for their determination, carcinogen−DNA adducts have become useful biomarkers of human exposure to genotoxic agents (*1*). These can be analyzed by immunological methods (for example, enzyme-linked immunosorbent assay, ELISA), biochemical methods (for example, ^{32}P-postlabeling), and physicochemical methods (for example, fluorescence spectroscopy and mass spectrometry) (*1*−*4*). However, techniques vary widely between laboratories, and there have been relatively few attempts to conduct interlaboratory comparisons to verify results (*5*−*7*).

What the field has lacked, and needs, is standards. Without these, it can be difficult to determine the efficiency with which adducts are detected by a particular analytical method in a particular laboratory. This has also made comparisons between results obtained in different laboratories difficult and uncertain. For DNA adduct measurements to be able to be applied to safety assessment and possibly for regulatory purposes, it is essential that research-based methods become more standardized and subjected to the rigors of quality assurance. To this end, the availability of validated standards can be of significant assistance. For a large interlaboratory trial of postlabeling methods, a number of such standards were prepared and analyzed in the participating laboratories and initially used to assist in the process of developing standardized protocols (*8*). In a subsequent stage of the trials, the standards were used as positive controls for the analysis of unknown samples, and for the normalized quantitation of adduct levels in the unknowns. The four standards that were prepared consisted of DNA modified by a polycyclic aromatic hydrocarbon (benzo[*a*]pyrene), by an aromatic amine (4-aminobiphenyl), by a heterocyclic amine (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, PhIP), and by a methylating agent (*N*-methyl-*N*-nitrosourea). The preparation and characterization of the 4-aminobiphenyl standard have been reported (*9*). Here we report the preparation, characterization, and stability of the methylated DNA standard.

* To whom correspondence should be addressed. E-mail: martino@icr.ac.uk and davidp@icr.ac.uk.

Experimental Procedures

Preparation of Methylated DNA. Calf thymus DNA, which had been purified by treating an aqueous solution with RNase and proteinase K (*10*), was supplied by F. Kadlubar (National Center for Toxicological Research, Jeffersonville, AR). Its purity was checked by its ultraviolet absorbance ratios: $A_{230}/A_{260} = 0.42$ and $A_{260}/A_{280} = 1.87$. 3- and 7-methyladenine and -guanine were from Sigma-Aldrich (Poole, U.K.); O^6-methyldeoxyguanosine was provided by P. D. Lawley. [^{3}H-*methyl*]-*N*-Methyl-*N*-nitrosourea (1.5 Ci/mmol) was supplied by NEN-Du Pont (Stevenage, U.K.). **Caution:** *This substance is probably a human carcinogen, and should be handled with due care.*

To 1.4 μg (20 μCi) of tritiated methylnitrosourea was added 80 μg of unlabeled methylnitrosourea in 20 mL of ethanol. This was added to 200 mg of DNA dissolved in 100 mL of 0.08 M sodium acetate (pH 6), and the mixture was kept at 37 °C for 1.5 h. A 50 μL sample of the mixture gave 7572 tritium counts/min, a counting efficiency of 34%. The DNA was precipitated with 250 mL of ethanol containing 3% ammonium acetate, washed, dried, and redissolved in 60 mL of water. Ten milliliters of 14% sodium acetate and 180 mL of ethanol were added to reprecipitate the DNA; it was washed twice with ethanol and twice with ether and dried in air to yield 160 mg of methylated DNA. This sample contained about 20 methyl groups per one million bases; DNA with different levels of modification was obtained by varying the amount of unlabeled methylnitrosourea that was added, or occasionally by adding an unmodified DNA solution before the final precipitation. The modification level was determined as described below (Analysis of DNA).

Most of the methylated DNA was stored at −80 °C as the solid or as a frozen solution (samples of 1 mg in 1 mL) in 5 mM BisTris + 0.1 mM EDTA buffer, pH 7. Other 1 mL samples were stored at room temperature, at 10 °C, −20 °C or under liquid nitrogen, to determine the long-term stability at various tem-

10.1021/tx990182e CCC: $19.00
Published on Web 03/18/2000

peratures. Samples were taken by weight or by volume of solution for analysis as described below.

Loss of Methylpurines. Methylated DNA (20 mg) was prepared as described above, containing one methyl group per 2000 bases, with a tritium activity of 1.4 μCi/g of DNA. It was dissolved in aqueous buffer solution [50 mM sodium phosphate (pH 7) and 4 mM sodium azide], and 50 μL each of 7-methylguanine and 3-methyladenine solutions (1 mg/mL) added as markers. The mixture was incubated at 10, 22, or 39 °C. Samples (0.5 mL) were taken, and DNA was precipitated by adding 1.3 mL of ethanol containing 3% ammonium acetate. The DNA was washed with ethanol and ether, dried, and analyzed as described below. The supernatant was evaporated at low pressure to about 0.2 mL, and then 0.4 mL of 0.05 M ammonium formate was added and the mixture filtered if necessary. It was analyzed on a Nucleosil ODS column (5 μm, 250 mm × 4.6 mm) eluted with 15% methanol in 0.04 M ammonium formate (pH ~6.5), at a rate of 0.8 mL/min. Fractions (1.5 min) were assayed for ultraviolet absorbance and tritium activity, to determine the amount of tritiated 7-methylguanine (eluted at ca. 10 min) and 3-methyladenine (ca. 20 min).

Analysis of DNA. Methylated DNA was hydrolyzed by treatment with enzymes: for 1 mL of solution (containing 1−2 mg of DNA), 20 μL of DNase 1 (Boehringer; 5 mg/mL) and 20 μL of buffer [0.5 M Tris (pH 7) and 0.5 M $MgCl_2$] for 17 h, 10 μL of snake venom phosphodiesterase (Sigma type VII, 3.3 units/mL) and 0.1 mL of buffer [0.5 M Tris (pH 9)] for 7 h, and 10 μL of alkaline phosphatase (Sigma type III, 300 units/mL) for 17 h, all at 37 °C. The resulting mixture was analyzed by liquid chromatography on an ODS column as described above, but eluted with a water/methanol gradient [0.05 M ammonium formate (pH 6.5) for 20 min, and then to 40% methanol over the course of 40 min, at a rate of 0.8 mL/min]. Fractions (2 min) were collected and assayed for ultraviolet absorbance and tritium activity.

The column separated deoxycytidine (17 min), 5-methyldeoxycytidine (37 min), deoxyguanosine and thymidine (together at 40 min), and deoxyadenosine (51 min); these were quantified by their ultraviolet absorption at 260 nm (extinction coefficients, by our estimates: dCyd, 7000; 5-Me-dCyd, 5000; dGuo and Thd, 10 000; and dAdo, 15 000). The molar ratios were close to the expected values (22:50:28 C:G/T:A). The methylpurines were released as the bases during hydrolysis, except for O^6-methyldeoxyguanosine; any other remaining methylpurine nucleosides, being positively charged, should be eluted by the aqueous starting solvent, but only 5−10% of the total tritium activity was recovered in this region. The retention times of 3-methylguanine, 7-methylguanine, 7-methyladenine, 3-methyladenine, and O^6-methyldeoxyguanosine were about 30, 40, 43, 46, and 58 min, respectively. They were quantified by their tritium activity, by adding scintillation fluid (Optiphase Safe, Fisher, Loughborough, U.K.) and counting in a Packard scintillation counter.

Quantitation of Methyl Groups in DNA. The efficiency of scintillation counting was estimated as follows. 7-Methylguanine with a lower specific activity was prepared by treating 1 mg of DNA with 2 mg (5 μCi) of methylnitrosourea, and then heating it (100 °C, 30 min). The resulting free 7-methylguanine was chromatographically isolated and quantified by both ultraviolet absorption (molar absorption coefficient of 7400 at 283 nm; *11*) and scintillation counting, and hence, the counting efficiency for this base was determined to be 35%. The occurrence of adducts in other samples of methylated DNA could then be estimated per one million bases as (counts per minute in product × millimoles of methylnitrosourea used)/[0.35(efficiency) × 2.22 × millicurie of methylnitrosourea used × millimoles of DNA].

Results

Preparation of Methylated DNA. Standard methylated DNA was prepared by treating calf thymus DNA with *N*-methyl-*N*-nitrosourea at pH 6−7, at a sufficient concentration to produce about seven methylated bases per one million nucleotides, and with tritium in the methyl groups to the minimum level needed for assay by radioactivity detection (0.5 μCi/g). Other samples were also prepared with higher levels of methylation. The 7-methylguanine:3-methyladenine:O^6-methylguanine:7-methyladenine ratio in the DNA was estimated to be 79:8:9:3. This is similar to that reported by Lawley and Shah (78:12:7.7:2.5; *12*).

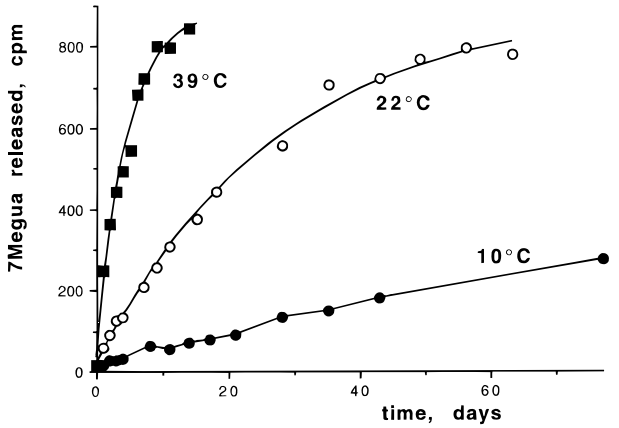

Figure 1. Release of 7-methylguanine from methylated DNA, as measured by its tritium radioactivity. The upper two curves are negative exponential curves with the data superimposed.

Table 1. Half-Lives of Methylpurines in DNA

	10 °C	22 °C	39 °C
half-life for release of 7Me-guanine (h)	3800	461	70
half-life for release of 3Me-adenine (h)	not determined	266	30

Rate of Release of Methylpurines from DNA. Methylated DNA (ca. one methyl group per 2000 bases) was incubated in aqueous buffer at neutral pH at 10, 22, or 39 °C, and samples were taken at various times. The DNA was precipitated, and the supernatant analyzed by liquid chromatography for 7-methylguanine and 3-methyladenine. These were identified by their coelution with added marker compounds, and quantified by their tritium radioactivity. The results are shown in Figure 1. The curves at 22 and 39 °C exhibit first-order kinetics, and that at 10 °C would probably exhibit the same if followed to completion. The half-lives were estimated using a programmable calculator procedure (*13*) and are given in Table 1.

The 7-methylguanine peak may also contain 7-methyladenine, but this should amount to only 2−3% of the 7-methylguanine and is released quickly, and would not affect the results.

The precipitated DNA was hydrolyzed to nucleosides with enzymes, and separated by liquid chromatography on a reverse-phase column, using a water/methanol gradient. A typical elution pattern is shown in Figure 2. Labile methylated nucleosides still remaining in the DNA lost deoxyribose during hydrolysis, and were eluted from the column as 7-methylguanine and 3-methyladenine at about 40 and 46 min. O^6-Methyldeoxyguanosine eluted at about 58 min.

The results of the stability study at 39 °C are shown in Figure 3. The amount of 7-methylguanine fell with time, as expected; the initial rate of fall was consistent

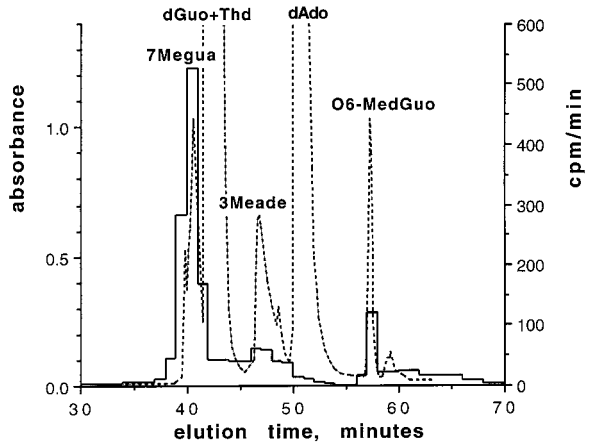

Figure 2. Elution of methylated DNA components from ODS, using an ammonium formate/methanol gradient. The methylated bases and nucleosides were located by reference to UV-absorbing markers, and quantified by their tritium radioactivity. The dotted line shows the elution of deoxyguanosine + thymidine and deoxyadenosine, and added 7-methylguanine, 3-methyladenine, and O^6-methyldeoxyguanosine markers; the 7-methylguanine was coincident with 5-methyldeoxycytidine from the DNA. The histogram shows tritium activity eluted per minute. The example that is shown is from methylated DNA after storage for 5 years at −20 °C.

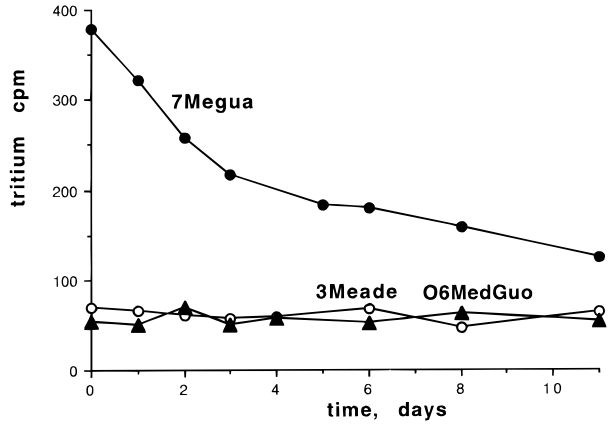

Figure 3. Change in content of methylated DNA during incubation at 39 °C and neutral pH.

with the half-life of 70 h estimated above, but the last four values were higher than expected. This discrepancy from first-order kinetics was not observed in the amount of 7-methylguanine that was released (Figure 1), suggesting a defect in the measurement of the amount of 7-methylguanine in the precipitated DNA. The sizes of the 3-methyladenine and O^6-methyldeoxyguanosine peaks were initially each about 15% of that of 7-methylguanine; they both remained constant with time. This was as expected for O^6-methyldeoxyguanosine, which is stable, but unexpected for 3-methyladenine, which should have disappeared within the first 5 days; the reason for its apparent persistence was unclear. It could have been due to a stable adduct which coeluted with 3-methyladenine, but the other known adducts are not eluted here.

In a separate experiment, DNA was precipitated from solutions containing 2 mg/mL DNA and 2−20 mg/L 7-methylguanine or 3-methyladenine, to see whether the DNA would carry the purine down with it. In each case, more than 90% of the methylpurine remained in solution.

Stability of O^6-Methylguanine in DNA. Another sample (10 mg) of this methylated DNA was dissolved

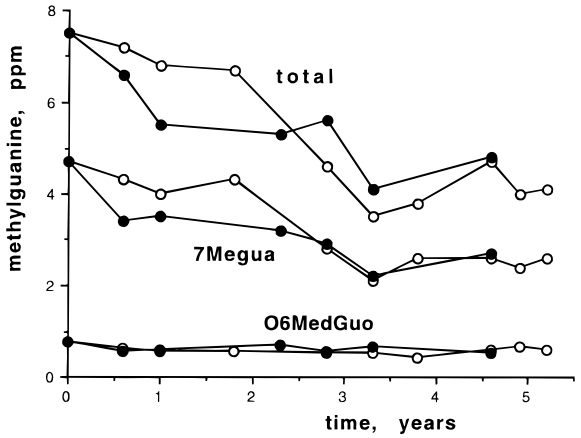

Figure 4. Loss of methylguanines from DNA on storage: (○) at −20 °C and (●) at −80 °C. The total methyl content (top curves) decreased from 7.5 to 4.0 per one million DNA bases in 5 years and the 7-methylguanine content (middle curves) from 4.7 to 2.4, while the O^6-methyldeoxyguanosine content did not change significantly (mean value of 0.59 ± 0.09 per one million bases, bottom curves). Analysis of DNA stored as a frozen solution at −190 °C for 0.6 or 2.3 years, or as a solid at −80 °C for 0.6 or 1 years, gave the same results as the frozen solution at −80 °C (data not shown).

in 5 mL of 0.05 M phosphate buffer (pH 7) and heated in a water bath at 89 °C. Samples (1 mL) were taken after 12, 30, 77, 160, and 230 min, and the DNA was isolated from each by adding 2.5 mL of ethanol containing 3% ammonium acetate, cooling, and centrifugation; it was no longer fibrous because of extensive strand breakage. Hydrolysis to nucleosides, followed by chromatographic separation, gave the normal nucleosides and tritium-labeled O^6-methyldeoxyguanosine. The 12 min sample yielded a small amount 7-methylguanine, but the other samples gave none of the labile methylated purines, as they are lost from the DNA within a few minutes. The amount of O^6-methyldeoxyguanosine remained constant, showing that this base is stably attached to the DNA, and that its tritium content is not easily exchanged with the solvent at neutral pH.

Stability of Frozen Methylated DNA. Methylated DNA was stored as a frozen solution (1 mg/mL) in BisTris/EDTA buffer, and kept at various temperatures for extended periods of time. Samples were precipitated to remove labile material, then hydrolyzed to nucleosides with enzymes, and analyzed on the reverse-phase column as described above. Some results are given in Figure 4. The results show a slow loss of radioactivity from the DNA, which is largely accounted for by loss of 7-methylguanine. The loss of 7-methylguanine proceeded with a half-life of about 4 years at −20 °C; it was only slightly slower (ca. 6 years) at −80 °C, which was somewhat surprising. The yield of O^6-methyldeoxyguanosine did not change significantly with time (95% confidence limits for the half-life at −20 °C were 12 years and infinity). The observed variations were probably due to difficulties in keeping the assay consistent over time, rather than any intrinsic variability.

Discussion

There are many agents which methylate DNA. To create a standard methylated DNA, we wanted an agent which would give a relatively high yield of O^6-methylguanine residues, as these are of the greatest biological

interest. We chose *N*-methyl-*N*-nitrosourea, where the proportion of methylation at O^6-guanine was known to be about 7%, and which was readily available as a tritium-labeled species. The range and proportion of products with this agent are closely similar to those seen in DNA isolated from the liver of rats treated with the environmental carcinogen *N*,*N*-dinitrosodimethylamine (*14*).

There are several good methods for analyzing methylated DNA (reviewed by Lawley in ref *15*). We chose hydrolysis by enzymes to nucleosides, followed by liquid chromatography, as this gave acceptable results for both the labile products (which were recovered as methylpurines) and the chief stable adduct, O^6-methyldeoxyguanosine. For the present purposes, we ignored the minor products 1-methyladenine, 3-methylguanine, methylated deoxycytidine and thymidine, and methyl phosphotriesters. The amounts of these can be estimated by other methods (*15*).

The methylated standard was stable on storage, except for the radioactive decay of the tritium label, and the hydrolytic cleavage of methylpurines from the DNA. The methylpurines remain in solution and can still be found by analysis, unless the DNA is precipitated before the assay, when they are lost in the supernatant. It appeared to make little difference whether the DNA was stored as a solid at −80 °C or as a frozen solution at −20, −80, or −190 °C.

In principle, the tritium label in the methyl groups may be subject to exchange with water. However, the high rate of recovery of tritiated O^6-methyldeoxyguanosine after 5 years, and the high rate of recovery of 7-methylguanine if the supernatant was included, indicate that less than 10% of the observed loss of methylated base was due to tritium exchange.

The rates of loss from DNA of the two major products, 7-methylguanine and 3-methyladenine, were best measured by precipitating the DNA, and estimating the quantity of these purines remaining in solution. In principle, it should be possible to measure the rates equally well by analyzing the precipitated DNA, but we found that this gave less accurate results, possibly because of interference by other products with unknown compositions. The rates given in Table 1 are therefore based on analysis of the purines released from DNA. The half-life for 7-methylguanine loss has been determined by other authors, as follows: 105 h at pH 7 and 37 °C (*16*), about 4.3 h at pH 8 and 65 °C (*17*), and 4.1 min at pH 7 and 100 °C (*18*); the rate decreases only slightly with pH (*19*). The corresponding rate constants are plotted in Figure 5, along with those obtained at lower temperatures in the work presented here. They fit the Arrhenius equation $\ln k\ (s^{-1}) = 27 - 12507/T$ (K), corresponding to an activation energy of 25 kcal/mol, and a trebling of the rate for each 10° rise in temperature. Extrapolation of the line would predict a half-life of about 80 years at −20 °C, but the data of Figure 4 indicate a half-life of 4 years in frozen solution. Storage in a liquid solution (e.g., a water/glycerol mixture at −20 °C) may therefore be a viable alternative to storage as the frozen solution.

The half-life for 3-methyladenine loss at pH 7 and 37 °C is 38 h (*16*); this is close to our estimate of 30 h at 39 °C. The minor product 7-methyladenine is lost with a half-life of only 2.8 h (*16*), so much of this product may have been lost during the preparation of the standard DNA samples.

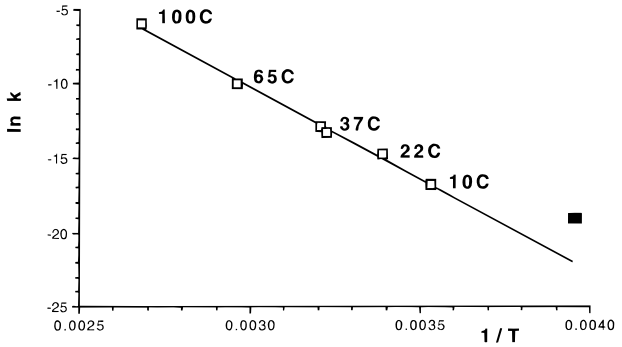

Figure 5. Effect of temperature on the loss of 7-methylguanine from DNA. Arrhenius plot of log(rate constant) (s^{-1}) vs reciprocal temperature (K). The rate in frozen solution at −20 °C is included as a black square.

Since O^6-methylguanine residues in DNA appear to be stable even at 89 °C, and there is no known mechanism for their loss from DNA in vitro, it may be assumed that the frozen methylated DNA standard will retain a constant level of O^6-methylguanine for many years. The other methylated bases are slowly cleaved from the DNA, and analysis of the 7-methylguanine or 3-methyladenine content must include the solution in which it is stored; otherwise, the estimates will slowly change with time.

The methylated standard was distributed to participants in an international interlaboratory trial of ^{32}P-postlabeling (*8*). A published method for determining the level of O^6-methylguanine in DNA (*20*) was tested on the DNA standard in participating laboratories without investigators knowing the exact level of modification. In the first trial, their mean value for the level of O^6-methylguanine was very close to the value given in this paper, provided that the results from those participants who failed to detect any adducts were excluded from the analysis (*8*). In a subsequent trial, an unknown sample was analyzed and the results were normalized against our DNA standard, the level of modification of the latter by now known to the investigators. The mean level calculated for the unknown sample by this procedure was found to be 50% higher than that calculated from the ^{3}H incorporation method. With the use of the methylated standard and also with the use of standards for DNA adducts of bulky carcinogens, the trials demonstrated that interlaboratory variability is reduced when results are normalized to values for validated standards that are analyzed concomitantly (*9*).

For investigators wishing to use a standard in their assays for methylated DNA adducts, the standard described here is available in 1 mg aliquots on application to the authors, while stocks last.

Acknowledgment. This work was supported by the Cancer Research Campaign, U.K., and by a grant from the European Union (Project EV5V-CT94-0449).

References

(1) Strickland, P. T., Routledge, M. N., and Dipple, A. (1993) Methodologies for measuring carcinogen adducts in humans. *Cancer Epidemiol., Biomarkers Prev.* **2**, 607−619.

(2) Weston, A. (1993) Physical methods for the detection of carcinogen-DNA adducts in humans. *Mutat. Res.* **288**, 19−29.

(3) Santella, R. M. (1988) Application of new techniques for the detection of carcinogen adducts to human population monitoring. *Mutat. Res.* **205**, 271−282.
(4) Phillips, D. H. (1990) Modern methods of DNA adduct determination. In *Chemical Carcinogenesis and Mutagenesis* (Cooper, C. S., and Grover, P. L., Eds.) Vol. 1 pp 503−546, Springer-Verlag, Berlin.
(5) Santella, R. M., Weston, A., Perera, F. P., Trivers, G. T., Harris, C. C., Young, T. L., Nguyen, D., Lee, B. M., and Poirier, M. C. (1988) Interlaboratory comparison of antisera and immunoassays for benzo[*a*]pyrene-diol-epoxide-I-modified DNA. *Carcinogenesis* **9**, 1265−1269.
(6) Savela, K., Hemminki, K., Hewer, A., Phillips, D. H., Putman, K. L., and Randerath, K. (1989) Interlaboratory comparison of the ^{32}P-postlabelling assay for aromatic DNA adducts in white blood cells of iron foundry workers. *Mutat. Res.* **224**, 485−492.
(7) Hemminki, K., Grzybowska, E., Chorazy, M., Twardowska-Sucha, K., Sroczynsky, J. W., Putman, K. L., Randerath, K., Phillips, D. H., Hewer, A., Santella, R. M., Young, T. L., and Perera, F. P. (1990) DNA adducts in humans environmentally exposed to aromatic compounds in an industrial area of Poland. *Carcinogenesis* **11**, 1229−1231.
(8) Phillips, D. H., and Castegnaro, M. (1999) Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. *Mutagenesis* **14**, 301−315.
(9) Beland, F. A., Doerge, D. R., Churchwell, M. I., Poirier, M. C., Schoket, B., and Marques, M. M. (1999) Synthesis, characterization, and quantitation of a 4-aminobiphenyl-DNA adduct standard. *Chem. Res. Toxicol.* **12**, 68−77.
(10) Gupta, R. C. (1993) ^{32}P-postlabelling analysis of bulky aromatic adducts. In *Postlabelling Methods for Detection of DNA Adducts* (Phillips, D. H., Castegnaro, M., and Bartsch, H., Eds.) pp 11−23, International Agency for Research on Cancer, Lyon, France.
(11) Lawley, P. D. (1971) The spectra of purines. In *Fused Pyrimidines Part II: Purines* (Lister, J. H., Ed.) pp 439−528, Wiley-Interscience, New York.
(12) Lawley, P. D., and Shah, S. A. (1973) Methylation of DNA by ^{3}H-^{14}C-methyl labelled *N*-methyl-*N*-nitrosourea: evidence for transfer of the intact methyl group. *Chem.-Biol. Interact.* **7**, 115−120.
(13) Osborne, M. R. (1982) A pocket calculator program to fit first-order kinetic curves. *Comput. Biol. Med.* **12**, 159−162.
(14) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, p 77, Plenum Press, New York.
(15) Lawley, P. D. (1976) Methylation of DNA by carcinogens: some applications of chemical analytical methods. In *Screening Tests in Chemical Carcinogenesis* (Montesano, R., Bartsch, H., and Tomatis, L., Eds.) Scientific Publication 12, pp 181−208, International Agency for Research on Cancer, Lyon, France.
(16) Lawley, P. D., and Warren, W. (1976) Removal of minor methylation products 7-methyladenine and 3-methylguanine from DNA of *Escherichia coli* treated with dimethyl sulphate. *Chem.-Biol. Interact.* **12**, 211−220.
(17) Haque, K., Cooper, D. P., van Delft, J. H. M., Lee, S. M., and Povey, A. C. (1997) Accurate and sensitive quantitation of N7-methyldeoxyguanosine-3′-monophosphate by ^{32}P-postlabeling and storage-phosphor imaging. *Chem. Res. Toxicol.* **10**, 660−666.
(18) Hemminki, K., Peltonen, K., and Vodicka, P. (1989) Depurination from DNA of 7-methylguanine, 7-(2-aminoethyl)guanine and ring-opened 7-methylguanines. *Chem.-Biol. Interact.* **70**, 289−303.
(19) Hendler, S., Fürer, E., and Srinivasan, P. R. (1970) Synthesis and chemical properties of monomers and polymers containing 7-methylguanine and an investigation of their substrate or template properties for bacterial DNA or RNA polymerases. *Biochemistry* **9**, 4141−4153.
(20) Povey, A. C., and Cooper, D. P. (1995) The development, validation and application of a ^{32}P-postlabelling assay to quantify O^6-methylguanine in human DNA. *Carcinogenesis* **16**, 1665−1669.

TX990182E