^{32}P-Postlabeling of *N*-(Deoxyguanosin-8-yl)arylamine Adducts: A Comparative Study of Labeling Efficiencies

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^{32}P-Postlabeling is an extremely powerful technique for the detection of DNA adducts. Typically, the quantitation of DNA adducts by ^{32}P-postlabeling is achieved by relative adduct labeling, via comparison of the radioactivity incorporated into the adducts to that associated with the normal nucleotides. This approach is based on a number of assumptions, the foremost being that normal and adducted nucleotide 3′-phosphates are converted to 3′,5′-bisphosphates with similar efficiencies. To evaluate labeling efficiencies for specific DNA adducts, we conducted a comparative study of the kinetics of phosphorylation by T_4 polynucleotide kinase using 2′-deoxyguanosine 3′-phosphate (dG3′p) and a series of *N*-(deoxyguanosin-8-yl)arylamine 3′-phosphate adduct standards (dG3′p-C8-Ar, Ar being 4-aminobiphenyl, 3- and 4-methylaniline, and 2,4- and 3,4-dimethylaniline). Phosphorylation of dG3′p and the dG3′p-C8-Ar adducts followed Michaelis−Menten kinetics. The apparent turnover numbers were 40−240-fold lower when labeling dG3′p-C8-Ar adducts compared to that when labeling dG3′p. The apparent specificity constant calculated for dG3′p-C8-4-aminobiphenyl (1.4 μM^{-1} min^{-1}) was approximately 4-fold lower than that (5.4 μM^{-1} min^{-1}) found for dG3′p. Apparent specificity constants for the monoarylamine adducts were even lower (0.043−0.23 μM^{-1} min^{-1}) and decreased in the following order: 4-methylaniline > 3,4-dimethylaniline > 3-methylaniline > 2,4-dimethylaniline. Similar experiments conducted with dG3′p-C8-Ar standards for 2-methylaniline and 2,3-dimethylaniline produced very poor and irreproducible labeling. These results indicate that ^{32}P-postlabeling of dG3′p-C8-Ar adducts is less efficient than that of dG3′p and suggest that normal nucleotides will be labeled preferentially to the arylamine adducts under kinetically controlled conditions. The data also indicate a further decrease in labeling efficiency upon substitution ortho to the amino group (e.g., 2,4-dimethylaniline). In addition, the ATP concentrations required for optimal labeling were found to be substantially higher than those used in typical ^{32}P-postlabeling assays. Since the high specific activity of carrier-free [γ-^{32}P]-ATP precludes increasing the ATP concentration to a significant extent, these data emphasize the need for using highly efficient adduct enrichment procedures when conducting ^{32}P-postlabeling analyses of DNA adducts.

Introduction

In the past two decades, ^{32}P-postlabeling (*1*, *2*) has become a widely used methodology for detecting carcinogen−DNA adducts. Although several improvements in the assay have appeared since its introduction, the basic procedure involves enzymatic hydrolysis of carcinogen-modified DNA to 3′-nucleotides, most often followed by an adduct enrichment step, subsequent 5′-^{32}P-phosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (PNK)[1], and chromatographic separation of carcinogen−nucleotide adducts from residual normal nucleotides prior to adduct detection and quantitation (*3*). By virtue of the high specific activity of [γ-^{32}P]ATP, the assay is extremely sensitive, and adduct levels as low as one in 10^{10} normal nucleotides have been reported using 5-10 μg of DNA (*3*). In addition, since the ^{32}P-postlabeling step does not rely on carcinogen-specific properties, the technique is remarkably versatile, being suitable for the detection of adducts from a wide variety of structurally distinct chemical carcinogens (*3*). This combination of high sensitivity and versatility has been instrumental in the emergence of ^{32}P-postlabeling as an important tool for monitoring human exposure to chemical carcinogens. Since chronic exposure to specific carcinogens appears to yield dose-related steady-state levels of DNA adducts in target tissues for tumorigenicity (*4*), the identification and accurate quantitation of human DNA adducts may be of great utility in estimating human cancer risk.

A major weakness of the ^{32}P-postlabeling methodology is that limited structural information is obtained. Selec-

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[1] Abbreviations: ABP, 4-aminobiphenyl; Ar, arylamine; dG3′p, 2′-deoxyguanosine 3′-phosphate; dG3′p-C8-Ar, *N*-(2′-deoxyguanosin-8-yl)-arylamine 3′-phosphate; dG3′,5′p, 2′-deoxyguanosine 3′,5′-bisphosphate; dG3′,5′p-C8-Ar, *N*-(2′-deoxyguanosin-8-yl)monoarylamine 3′,5′-bisphosphate; diMeA, dimethylaniline; dT3′p, thymidine 3′-phosphate; dTT, dithiothreitol; MeA, methylaniline; PEI, polyethyleneimine; PNK, T_4 polynucleotide kinase; RAL, relative adduct labeling.

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tive chemical or enzymatic tests [e.g., the susceptibility of arylamine 3′-nucleotide adducts to nuclease P_1 (*5*, *6*)], as well as the chromatographic behavior in selected solvents, may suggest a particular class of chemical carcinogens from which an unknown adduct is derived. However, although such tests form the basis of group-specific detection procedures (*3*), the assignment of adduct structures cannot be accomplished in the absence of standards.

One additional concern about ^{32}P-postlabeling is the accuracy of reported measurements of DNA adduct levels. Typically, the quantitation of DNA adducts is achieved by relative adduct labeling (RAL), in which the ratio of the adduct-associated radioactivity to the total nucleotide radioactivity is corrected using an appropriate dilution factor (*7*). Absolute levels of binding are often reported using this procedure, by assuming an average concentration of 3×10^6 fmol of nucleotides/μg of DNA (*8*). However, this approach is based on a number of *potentially* incorrect assumptions, the foremost being that adducted and normal nucleotide 3′-phosphates are converted to 3′,5′-bisphosphates with equal efficiency. Although reports about the quantitative aspects of the PNK-catalyzed phosphorylation step are relatively scarce, there is sufficient evidence that the labeling efficiency is adduct-dependent. For instance, some aromatic (*9*) and bulky (*10*) adducts appear to be better substrates for PNK than normal nucleotides, a property underlying the use of ATP-deficient postlabeling strategies. By contrast, other bulky and nonbulky adducts seem to be labeled with low efficiencies (*11*–*16*).

Aromatic amines and amides are chemical carcinogens to which humans are exposed from a variety of sources, including tobacco smoke (*17*–*21*). These carcinogens have been implicated in human bladder carcinogenesis (*22*) and are thought to play a role in the onset of bladder cancer in smokers (*22*–*24*). It has been suggested that aromatic amines may be also involved in the induction of breast cancer in postmenopausal women who smoke (*25*), and recently, single-ring aromatic amines have been reported to be present in human breast milk (*26*). Analyses of exfoliated bladder cells and autopsy and biopsy samples of human bladder (*27*–*30*) have indicated the presence of several putative arylamine–DNA adducts, among which only *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl has been identified (*28*–*30*). *N*-(Deoxyguanosin-8-yl)arylamine adducts are the major persistent adducts formed upon metabolic activation of aromatic amine carcinogens (*31*–*33*), and in recent work, we have demonstrated that these are also the major adducts from the in vitro reaction of DNA with electrophilic derivatives of alkylanilines present in tobacco smoke (*34*, *35*). Although the presence of alkylaniline-derived DNA adducts in human samples has yet to be established, hemoglobin–alkylaniline adducts have been detected, with the adduct levels from some of the alkylanilines being higher in smokers (*36*, *37*).

The quantitation of ^{32}P-postlabeled arylamine-modified DNA adducts using the RAL method seems to underestimate adduct levels in a consistent manner. For instance, when DNA adducts were assessed in mice treated with radiolabeled 2-acetylaminofluorene as part of an interlaboratory trial of ^{32}P-postlabeling procedures, the values obtained by RAL were 4–8-fold lower than those determined on the basis of ^{3}H incorporation (*38*). More recently, we reported a comparative adduct quantitation study of DNA samples modified in vitro and in vivo with 4-[^{3}H]aminobiphenyl, using different DNA adduct detection methodologies (*39*). The results obtained by ^{32}P-postlabeling consistently indicated DNA adduct levels that were 3–5% of the values determined by measuring the extent of ^{3}H incorporation. Although other factors, such as incomplete recovery of adducts during the hydrolysis and enrichment steps, may contribute to the underestimation of arylamine–DNA adduct levels by RAL, the intrinsic affinities of the adducts for PNK could play a major role in the process.

With the aim of evaluating relative labeling efficiencies of arylamine–DNA adducts, this study describes a comparative kinetic analysis of the PNK-catalyzed phosphorylation, using 2′-deoxyguanosine 3′-phosphate (dG3′p) and a number of *N*-(deoxyguanosin-8-yl)arylamine 3′-phosphate adduct standards (dG-3′p-C8-Ar, Ar being 4-aminobiphenyl, 3- and 4-methylaniline, and 2,4- and 3,4-dimethylaniline) derived from arylamines present in tobacco smoke.

Materials and Methods

Caution: *N-Arylamines, N-hydroxyarylamines, and N-acetoxyarylamines are potentially carcinogenic. They should be handled with protective clothing, in a well-ventilated fumehood. Exposure to ^{32}P should be kept as low as possible, by working in a confined laboratory area, with protective clothing, plexiglass shielding, Geiger counters, and body dosimeters. Wastes must be discarded according to appropriate safety procedures.*

Chemicals. 2′-Deoxyguanosine 3′-phosphate (dG3′p), thymidine 3′-phosphate (dT3′p), and apyrase (11.8 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). ATP was acquired from Boehringer Manheim Gmbh (Indianapolis, IN). [γ-^{32}P]ATP was synthesized from ADP (Sigma) and carrier-free, HCl-free [^{32}P]orthophosphate (ICN Pharmaceuticals, Costa Mesa, CA) (*2*, *8*). The [γ-^{32}P]ATP was diluted 500–1000-fold before being used in the labeling reactions. PNK (90 090 units/mg, 35 000 mg/mmol of monomer) was purchased from Amersham U.S. Biochemical (Cleveland, OH) at a concentration of 30 units/μL. Dilutions of the stock solution were performed as needed.

N-(2′-Deoxyguanosin-8-yl)-4-aminobiphenyl 3′-phosphate (dG3′p-C8-4ABP) and the *N*-(2′-deoxyguanosin-8-yl)monoarylamine 3′-phosphate adducts (dG3′p-C8-Ar, Ar being 3MeA, 4MeA, 2,4diMeA, and 3,4diMeA) were synthesized by reaction of the corresponding *N*-acetoxyarylamines with dG3′p and purified by reversed-phase HPLC as described previously (*34*). Adduct concentrations were determined by UV spectroscopy, using the extinction coefficients of the corresponding nucleoside adducts (*34*, *35*, *40*). All other chemicals and biochemicals were of analytical grade and were used without further purification.

Instrumentation. UV spectra were recorded with a Beckman DU-40 UV/vis spectrophotometer. Reaction mixtures were incubated at 37 °C in 150 μL polypropylene Eppendorf tubes, using a multiblock heater (Lab-Line Instruments, Inc., Melrose Park, IL). Liquid scintillation counting was performed on a Packard Tri-Carb model 1600TR instrument (Packard Instrument Co., Meriden, CT), using Ultima Gold (Packard) as the scintillation fluid. TLC plates were exposed to intensifying screens at room temperature for variable periods of time (1–12 h) in Kodak X cassettes. Visualization and quantitation of the chromatograms were then conducted with a phosphorimager (Storm 860 Imager, Molecular Dynamics, Sunnyvale, CA).

Chromatography. PEI-cellulose TLC plates, manufactured by Macherey-Nagel or Merck, were purchased from Alltech Associates, Inc. (Deerfield, IL). The Macherey-Nagel plates were washed with deionized water and dried prior to use. Specific chromatographic conditions were as follows.

(1) dG3′,5′p. The samples were applied to Merck plates (length of 20 cm), and the elution was conducted overnight onto a Whatman #1 paper wick using 150 mM sodium phosphate (pH 6.0).

(2) dG3′,5′p-C8-4ABP. The samples were applied to Macherey-Nagel plates (length of 10 cm). The elution was conducted on a Whatman #1 paper wick (direction D1) with 900 mM sodium phosphate (pH 6.8), and then in the opposite direction (D2) with 3.6 or 5.3 M lithium formate and 8.5 M urea (pH 3.5).

(3) dG3′,5′p-C8-Ar (Ar being 3MeA, 4MeA, 2,4diMeA, and 3,4diMeA). The samples were applied to Macherey-Nagel plates (length of 20 cm). The elution was conducted onto a Whatman #1 paper wick (direction D1) with 2.5 M sodium phosphate (pH 6.0), and then in the opposite direction (D2) with 1.8 M lithium formate and 4.2 M urea (pH 3.5).

Kinetic Studies. (1) Phosphorylation as a Function of Time. The time dependence of the conversion of dG3′p to the corresponding bisphosphate (dG3′,5′p) was determined by an adaptation of a literature procedure (*41*). A stock labeling solution (55 μL) containing PNK (60 milliunits/μL, 275 fmol) and ATP (16.1 μM, 886 pmol) in 80 mM bicine-NaOH, 40 mM $MgCl_2$, 40 mM dithiothreitol (dTT), 4 mM spermidine (pH 9.4) buffer was prepared. A 35 μL aliquot of this labeling solution was added to 105 μL of a 6.67 μM aqueous solution of dG3′p (700 pmol, 5 μM final concentration). The mixture was vortexed, centrifuged for 5−10 s, and incubated at 37 °C. Following incubation periods of 2, 5, 10, 20, 30, 60, and 120 min, 20 μL aliquots of the reaction mixture were removed, the reaction was quenched by mixing with 2 μL of 673 μM dT3′p (1346 pmol, 57.3 μM final concentration) and 30 milliunits of apyrase (1.5 μL), and the mixtures were further incubated for 30 min at 37 °C. Aliquots of each mixture (15 μL) were then spotted at 2 cm intervals on a 20 cm × 20 cm PEI-cellulose TLC plate and eluted as described above. Control incubations were conducted for 120 min in the absence of dG3′p, either with or without 100 pmol of dT3′p.

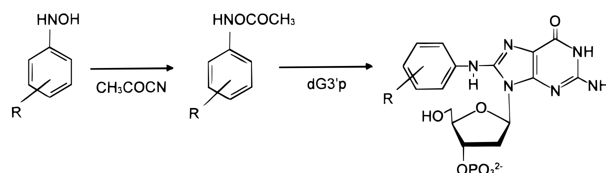
The time dependence of the dG3′p-C8-4ABP phosphorylation was studied in a similar manner. With this substrate, three independent experiments were performed, using 27.5, 275, and 2750 fmol of PNK (6, 60, and 600 milliunits/μL, respectively) in the stock labeling solution.

(2) Phosphorylation as a Function of ATP Concentration. The effect of ATP concentration on the initial velocity of the phosphorylation reaction was studied for the conversion of dG3′p and dG3′p-C8-4ABP to the corresponding bisphosphates. Several labeling mixtures (10 μL) were prepared, each containing 500 fmol of PNK (600 milliunits/μL) and different ATP concentrations in 80 mM bicine-NaOH, 40 mM $MgCl_2$, 40 mM dTT, 4 mM spermidine (pH 9.4) buffer. The total ATP concentrations were 4−2000 μM (0.04−20 nmol) for experiments conducted with dG3′p and 200−6800 μM (2−68 nmol) for experiments conducted with dG3′p-C8-4ABP. Following a 10 min equilibration at 37 °C, each labeling mixture was added to a preincubated 30 μL aqueous solution containing 200 pmol of the nucleotide substrate. The mixtures were then incubated for 5 min and treated subsequently as described for the time dependence study.

(3) Phosphorylation as a Function of Substrate Concentration. The effect of substrate concentration on the phosphorylation reaction was studied for dG3′p, dG3′p-C8-4ABP, and a variety of dG3′p-C8-Ar adducts (Ar being 3MeA, 4MeA, 2,4diMeA, and 3,4diMeA) using the methodology described above. The labeling solution contained 600 milliunits/μL (50 fmol) PNK and 406 μM (dG3′p) or 955 μM (arylamine adducts) ATP in 80 mM bicine-NaOH, 40 mM $MgCl_2$, 40 mM dTT, 4 mM spermidine (pH 9.4) buffer. Aliquots (5 μL) of this solution were added to 15 μL of various substrate solutions (typically 2−3600 pmol, 0.13−240 μM), and the mixtures were incubated and subsequently processed.

(4) Phosphorylation as a Function of PNK Concentration. The effect of PNK concentration on the initial velocity of the phosphorylation reaction was analyzed for the conversion of dG3′p and dG3′-C8-4ABP to the corresponding bisphosphates using the assay conditions described for the substrate concentration dependence study. Control incubations were performed in the absence of PNK.

Scheme 1. Synthesis of dG3′p-C8-Ar Adducts[a]



[a] R is 3-methyl, 4-methyl, 2,4-dimethyl, 3,4-dimethyl, or 4-phenyl.

(5) Calculations. The specific activity of the [γ-^{32}P]ATP in each labeling mixture was determined by liquid scintillation counting of diluted aliquots, whose concentration had been established by UV spectroscopy. Following elution, and prior to exposure of the TLC plate to the intensifying screen, an aliquot of the labeling mixture was spotted onto the plate to serve as a quantitation standard. Whenever necessary, corrections for ^{32}P decay were introduced in the calculations.

The kinetic parameters (V_{max} and K_m) were calculated using the weighted least-squares fitting (relative weights) program Leonora (*42*). Similar results were obtained with version 1.0 of the kinetics program developed by Brooks (*43*) by excluding outliers from the initial data sets and recalculating the kinetic parameters as needed.

Results

The goal of this work was to compare the kinetics of phosphorylation of dG3′p and a number of dG3′p-C8-Ar (Ar being 4ABP, 3MeA, 4MeA, 2,4diMeA, and 3,4diMeA) adducts from arylamines present in tobacco smoke under experimental conditions similar to those used in typical ^{32}P-postlabeling protocols. Therefore, the PNK-catalyzed phosphorylation reactions were conducted at 37 °C and pH 9.4, in a bicine-NaOH buffer containing $MgCl_2$, dTT, and spermidine (*3*). All the adducts were synthesized by reaction of the corresponding *N*-acetoxyarylamines with dG3′p (Scheme 1), as described in detail in our previous work (*34*, *35*).

The characterization of an enzyme-catalyzed reaction usually includes the determination of the maximum initial reaction velocity (V_{max}) and Michaelis constant (K_m) for each substrate. To obtain satisfactory estimates of these parameters, some preliminary experiments were necessary to ensure that the basic assumptions underlying the Michaelis−Menten relationship were observed. Specifically, an incubation time had to be selected within a range where the measured initial velocity was constant with time and varied linearly with the enzyme concentration (*44*). In addition, suitable chromatographic conditions had to be established for each substrate.

Chromatography. The elution conditions for the separation of dG3′,5′p and dG3′,5′p-C8-4ABP from the residual [γ-^{32}P]ATP and inorganic phosphate were adapted from literature procedures (*3*, and references therein). Due to the low lipophilicity of dG3′,5′p, a single elution in a phosphate buffer with a low ionic strength was sufficient to achieve substantial migration of the normal nucleotide (not shown). By contrast, a second elution with a lithium formate buffer containing 8.5 M urea was necessary to promote migration of the lipophilic dG3′p,5′p-C8-4ABP (Figure 1a). To prevent overmigration of the less lipophilic adducts (i.e., those derived from methylated anilines), a phosphate buffer with a high ionic

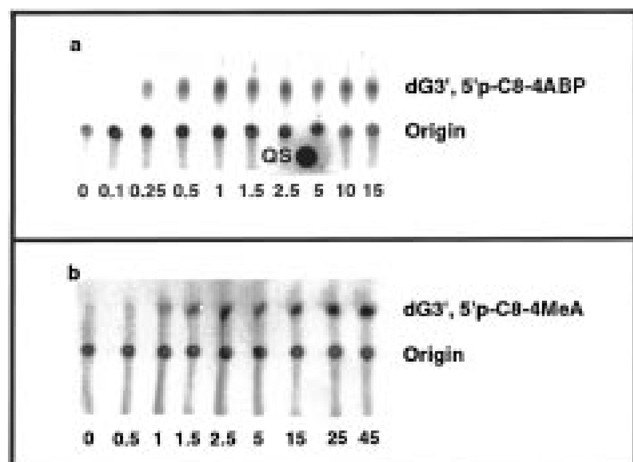


Figure 1. Representative TLC chromatograms for the phosphorylation of dG3′p-C8-4ABP (a) and dG3′p-C8-4MeA (b). The elution conditions are outlined in Materials and Methods. The substrate concentrations (micromolar) are denoted. QS indicates the quantitation standard.

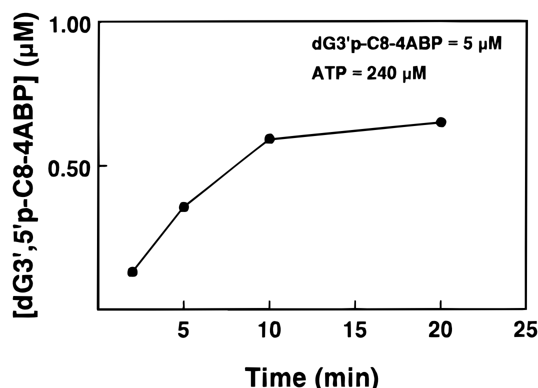


Figure 2. Expanded region (0−20 min) of the time course curve for the phosphorylation of dG3′p-C8-4ABP. The incubation conditions are outlined in Materials and Methods.

strength was used for D1, whereas the urea and lithium formate concentrations were decreased in the D2 buffer (Figure 1b), compared to those used for elution of dG3′,5′p-C8-4ABP (Figure 1a). This type of strategy is normally successful for the analysis of adducts containing less than two aromatic rings (*45*, *46*).

Selection of the Incubation Time. The time course of the PNK-catalyzed phosphorylation reaction was analyzed for the period from 0 to 120 min using either 5 μM dG3′p or dG3′p-C8-4ABP as the substrate and 15 milliunits/μL (1.2 nM) PNK. Both species underwent fast phosphorylation during the first 20−30 min. This was followed by a decline in the rate of substrate conversion as time progressed, with the product concentration tending toward a plateau. In both instances, no decrease in the concentration of the bisphosphate product was detected during the time of observation. This contrasts with a report by Hemminki et al. (*13*), who detected a decline in the concentration of dG3′,5′p-C8-4ABP after approximately 45 min, presumably due to a time-dependent dephosphorylation of the adduct. Regardless of the substrate, the measured velocity of phosphorylation was constant with time up to approximately 10 min, as illustrated for dG3′p-C8-4ABP (Figure 2). Similar results were obtained for this substrate at two additional PNK concentrations (0.12 and 12 nM, not shown). Since the linear region of the time course curves spanned approximately the same interval (0−10 min) for both dG3′p and dG3′p-C8-4ABP, an incubation time of 5 min was selected to conduct the subsequent experiments with these substrates and the remaining dG3′p-C8-Ar adducts.

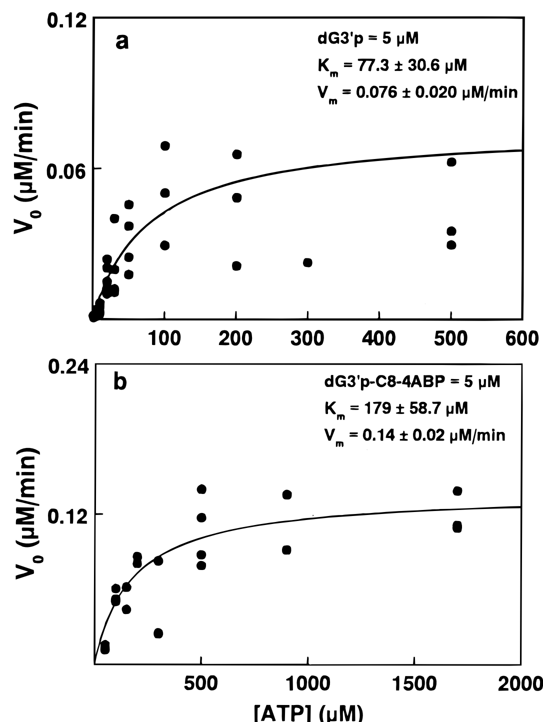


Figure 3. Variation of the initial velocity of phosphorylation as a function of the ATP concentration using (a) 5 μM dG3′p and (b) 5 μM dG3′p-C8-4ABP as the substrate. The incubation conditions are outlined in Materials and Methods.

Selection of the PNK Concentration. The initial velocity of the dG3′p phosphorylation reaction was found to vary linearly with the PNK concentration from 25 to 800 milliunits/μL (2.0−64 nM). Similar results were obtained for the phosphorylation of dG3′p-C8-4ABP. Since the accumulation of bisphosphate products had to be avoided to maintain stationary-state conditions, the PNK concentration was selected to be approximately 2 orders of magnitude lower than the range of substrate concentrations to be used in the kinetic measurements. Therefore, all the subsequent experiments were conducted using 150 milliunits/μL (12 nM) PNK in the incubation mixture. This enzyme concentration lies within the range of PNK concentrations (100−1000 milliunits/μL) typically used in ^{32}P-postlabeling protocols (*3*, *47*).

Selection of the ATP Concentration. The effect of the ATP concentration on the initial velocity of the phosphorylation reaction was studied using dG3′p and dG3′p-C8-4ABP at a concentration of 5 μM. The experimental conditions were adapted from those reported by Lillehaug and Kleppe (*41*) and from typical ^{32}P-postlabeling protocols (*3*, and references therein), taking into account the preliminary time course and enzyme linearity tests described above. With both substrates, more data points were obtained in the low-ATP concentration region to achieve better accuracy in the definition of the experimental curves (*42*). The results are displayed in Figure 3 (panels a and b), and the calculated kinetic parameters are shown in Table 1. The analysis of both curves indicates that Michaelis−Menten kinetics occurred; however, it is clear that a higher ATP concentra-

Table 1. Kinetic Parameters Obtained for ATP Using Different Substrates

substrate[a]	K_m (μM)[b]	V_{max} (μM min^{-1})[b]	k_{cat} (min^{-1})[c]	k_{cat}/K_m (μM^{-1} min^{-1})[d]
dG3′p	77.3 ± 30.6	0.076 ± 0.020	6.39	0.083
dG3′p-C8-4ABP	179 ± 58.7	0.14 ± 0.02	11.5	0.065

[a] The substrate concentration was 5 μM. [b] Calculated for the Michaelis–Menten equation using the weighted least-squares (relative weights) fitting program Leonora (*42*). Both K_m and V_{max} are apparent values and are presented ± the standard error. [c] Apparent turnover number, defined as V_{max}/[E$_0$]. The PNK concentration in the incubation mixture was 150 milliunits/μL (12 nM). [d] Apparent specificity constant.

tion was required to reach the apparent V_{max} plateau when using dG3′p-C8-4ABP as a substrate. This is reflected in the higher apparent K_m value (179 μM) calculated for ATP in the presence of dG3′p-C8-4ABP, as compared to that (77.3 μM) obtained in the presence of dG3′p. Interestingly, the apparent V_{max} was higher when dG3′p-C8-4ABP was used as a substrate, which resulted in a higher apparent turnover number (k_{cat}; Table 1). Nonetheless, within experimental error, the apparent specificity constant (k_{cat}/K_m; Table 1) for ATP appeared to be unaffected by the type of nucleotide substrate (i.e., normal vs adducted dG3′p). In view of the results obtained for the apparent K_m constants, subsequent experiments to establish the kinetic parameters for the phosphorylation of dG3′p were conducted using 101 μM ATP, whereas all the incubations conducted with dG3′p-C8-Ar adducts were performed using 240 μM ATP. Although not strictly corresponding to ATP-saturating conditions, the selected ATP concentrations were higher than the apparent K_m constants listed in Table 1 and provided satisfactory kinetic data without an excessive increase in the amount of ^{32}P handled in each incubation.

Determination of the Kinetic Parameters of Phosphorylation for dG3′p and the dG3′p-C8-Ar Adducts. Representative curves displaying the variation of the initial velocity of phosphorylation (incubation time of 5 min) as a function of substrate concentration are shown in Figure 4 for dG3′p and two of the dG3′p-C8-Ar adducts (Ar being 4ABP and 3,4diMeA). Substrate concentrations in the incubation mixtures ranged from 0.1 μM to as high as 180 μM, with the higher concentration dictated in each case by the need to reach the V_{max} plateau. Similar to the case in the ATP dependence study, more data points were obtained in each experiment in the region of low substrate concentration (Figure 4). The kinetic parameters calculated for all the substrates are shown in Table 2. In every instance, the phosphorylation reaction appeared to follow Michaelis–Menten kinetics. In addition to the substrates listed in Table 2, similar experiments were conducted using dG3′p-C8-Ar adduct standards derived from 2-methylaniline and 2,3-dimethylaniline. Very poor and irreproducible labeling was obtained with these two substrates, which precluded the calculation of reliable kinetic parameters.

The data in Table 2 indicate apparent K_m values ranging from 0.86 μM for dG3′p-C8-4ABP to 20.4 μM for dG3′p-C8-2,4diMeA, with the normal nucleotide dG3′p exhibiting an intermediate apparent K_m (10.5 μM). This suggests that a higher substrate concentration is required to reach V_{max} when labeling dG3′p, compared to most of the adducts investigated in this study. However, the apparent V_{max} was found to be much greater with dG3′p than with any of the adducted substrates, leading to a greater extent of substrate conversion during the incubation period. This is clearly reflected in the apparent turnover numbers (k_{cat}), which were 40–240-fold lower

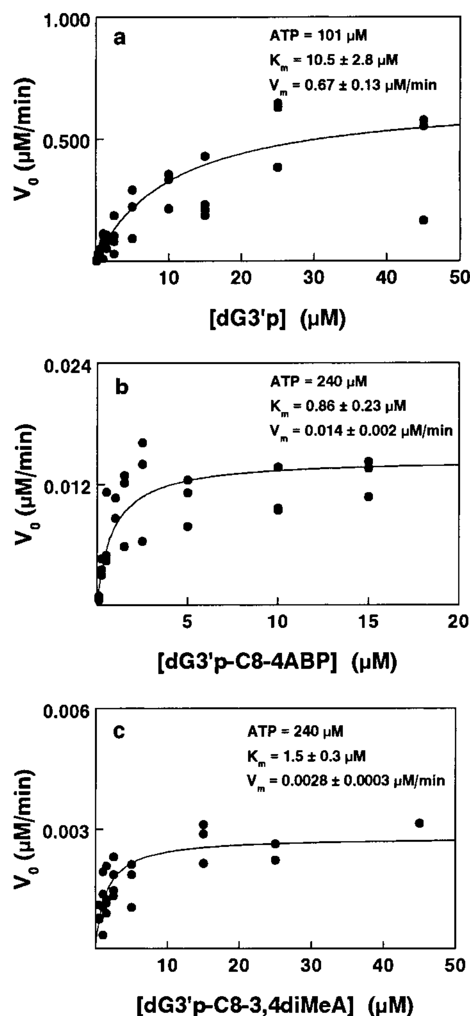


Figure 4. Variation of the initial velocity of phosphorylation as a function of the substrate concentration using (a) dG3′p as the substrate, in the presence of 101 μM ATP, (b) dG3′p-C8-4ABP as the substrate, in the presence of 240 μM ATP, and (c) dG3′p-C8-3,4-diMeA as the substrate, in the presence of 240 μM ATP. The incubation conditions are outlined in Materials and Methods.

when labeling the dG3′p-C8-Ar adducts than when labeling dG3′p (Table 2). Moreover, the apparent enzyme specificity constant (k_{cat}/K_m) was approximately 4-fold lower for dG3′p-C8-4ABP and 20–130-fold lower for the monoarylamine adducts compared to that for dG3′p. These findings strongly suggest that ^{32}P-postlabeling of dG3′p-C8-Ar adducts is less efficient than that of dG3′p, and therefore, kinetically controlled conditions are likely to lead to preferential labeling of normal nucleotides, whenever these are present in the incubation mixture. In addition, the lowest labeling efficiency was found for adducts containing a substituent ortho to the arylamine nitrogen. This is inferred from the small apparent PNK specificity constant (0.043 μM^{-1} min^{-1}; Table 2) deter-

Table 2. Kinetic Parameters Obtained for dG3′p and dG3′p-C8′-Ar Adducts

substrate[a]	K_m (μM)[b]	V_{max} (μM min^{-1})[b]	k_{cat} (min^{-1})[c]	k_{cat}/K_m (μM^{-1} min^{-1})[d]
dG3′p	10.5 ± 2.8	0.67 ± 0.13	56.6	5.38
dG3′p-C8-4ABP	0.86 ± 0.23	0.014 ± 0.002	1.22	1.41
dG3′p-C8-3MeA	5.3 ± 1.0	0.0060 ± 0.0007	0.51	0.095
dG3′p-C8-4MeA	5.6 ± 1.8	0.015 ± 0.003	1.30	0.232
dG3′p-C8-2,4diMeA	20.4 ± 6.1	0.010 ± 0.002	0.87	0.043
dG3′p-C8-3,4diMeA	1.5 ± 0.3	0.0028 ± 0.0003	0.24	0.156

[a] Substrate concentrations ranged from 0.1 to 180 μM in the incubation mixtures. The ATP concentration was 101 (dG3′p) or 240 μM (arylamine adducts). [b] Calculated for the Michaelis−Menten equation using the weighted least-squares (relative weights) fitting program Leonora (*42*). Both K_m and V_{max} are apparent values and are presented ± the standard error. [c] Apparent turnover number, defined as V_{max}/[E$_0$]. The PNK concentration in the incubation mixture was 150 milliunits/μL (12 nM). [d] Apparent specificity constant.

mined with dG3′p-C8-2,4diMeA, and also from the irreproducible labeling obtained when the 2-methylaniline and 2,3-dimethylaniline standards were used as substrates.

Discussion

In this study, we compared the kinetics of the PNK-catalyzed phosphorylation of nucleotide 3′-monophosphates, using dG3′p and a variety of dG3′p-C8-Ar adducts (Ar being 4ABP, 3MeA, 4MeA, 2,4diMeA, and 3,4diMeA) derived from arylamines present in tobacco smoke. dG3′p-C8-4ABP was selected for analysis because 4-aminobiphenyl is considered to be a human bladder carcinogen (*22*) and dG3′p-C8-4ABP has been identified in human DNA (*28−30*). Presently, it is not known if adducts derived from alkylanilines are part of the putative arylamine−DNA adducts detected in human samples (*27−30*). Although, as a class, single-ring aromatic amines are considered weak carcinogens (*48*), some (e.g., 2-methylaniline and 2,6-dimethylaniline) are rodent (*49−51*) and possibly human (*51*, *52*) carcinogens. Since alkylated anilines are major contributors to the aromatic amine fraction of cigarette smoke (*18−20*) and because their electrophilic derivatives can bind to DNA (*34*, *35*), it is important to determine if typical ^{32}P-postlabeling conditions are adequate for the detection and quantitation of alkylaniline−DNA adducts.

Kinetic parameters for the phosphorylation of normal nucleotide 3′-monophosphates have not been reported, with the exception of a study by Lillehaug and Kleppe (*41*), who obtained an apparent K_m of 22.2 μM for dT3′p in the presence of 66 μM ATP. Preliminary work on the phosphorylation of dG3′p-C8-4ABP was conducted by Hemminki and co-workers (*13*, *15*), but K_m or V_{max} values were not obtained.

We performed initial experiments using dG3′p and dG3′p-C8-4ABP as substrates, to establish incubation times, as well as PNK and ATP concentrations suitable for estimating the kinetic parameters. In the labeling step, we used a bicine-NaOH buffer containing $MgCl_2$, dTT, and spermidine, which allows the maintenance of a high pH (ca. 9−9.5). Although optimum labeling of normal and adducted nucleotides has been reported to occur at pH 7.4−8.4 (*53*), most ^{32}P-postlabeling assays are conducted under alkaline conditions with the aim of minimizing the 3′-phosphatase activity of PNK (*54*). Since our goal was to compare the kinetics of phosphorylation of dG3′p and dG3′p-C8-Ar adducts under conditions typical of current ^{32}P-postlabeling protocols, an alkaline pH was maintained in all incubations. Regardless of the substrate type (i.e., normal vs adducted dG3′p), our initial time dependence measurements indicated that the phosphorylation step is fast, with the product concentration tending toward a plateau after 20−30 min. This is in good agreement with the results of Hemminki and co-workers for dG3′p-C8-4ABP (*13*), although we were unable to detect a subsequent decline in the concentration of the bisphosphate product reported by those authors. This may be due to differences in labeling conditions because Hemminki et al. (*13*) conducted their incubations at pH 7.9, which would favor phosphatase activity compared to the pH 9.4 used in our study. In any case, since the PNK-catalyzed phosphorylation is fast and because dG-C8-Ar adducts are known to be unstable under alkaline conditions (*55*), it is apparent that long incubation times can and should be avoided when quantifying this type of adduct by ^{32}P-postlabeling.

Using dG3′p and dG3′p-C8-4ABP as substrates, we found the initial velocity of phosphorylation to be linearly dependent on PNK over a broad range of enzyme concentrations. The value selected to conduct the kinetic studies (150 milliunits/μL) was sufficiently low to prevent accumulation of bisphosphate products and was well within the range used in typical ^{32}P-postlabeling protocols (*3*, *47*). By contrast, whereas typical ^{32}P-postlabeling protocols use ca. 2−3 μM [γ-^{32}P]ATP (*3*, *47*), the apparent K_m values obtained for ATP with dG3′p and dG3′p-C8-4ABP as substrates (77.3 and 179 μM, respectively; Table 1) suggest that optimal labeling requires ATP concentrations much higher than those normally used. A similar observation has been reported by Reddy and co-workers (*56*), who demonstrated that increasing the concentration of ATP to 60 μM resulted in maximal labeling of thymine glycol adducts. If we assume that our results with dG3′p-C8-4ABP can be extrapolated to other dG3′p-C8-Ar adducts, the requirement for higher ATP concentrations may be even more critical for the adducts than for normal nucleotides.

On the basis of these results, 240 μM ATP was used in the incubations to establish the kinetic parameters of the arylamine adducts. This compares to the 101 μM ATP used in the incubations conducted with dG3′p. These ATP concentrations were lower than those (*44*) required for saturation, and as a result, all the kinetic parameters listed in Table 2 must be regarded as apparent values. Nonetheless, of the parameters presented in Table 2, the apparent specificity constants (k_{cat}/K_m) are the most useful for comparative purposes. Since specificity constants can be used in reactions with two substrates under nonsaturating concentrations (*57*), we chose not to increase the ATP concentrations to minimize exposure to ^{32}P.

In all instances (see Figure 4 for representative examples), the phosphorylation reactions followed Michae-

lis–Menten kinetics. Taking into account the fact that the active form of PNK is a tetramer (*58*), we also used Hill's equation to treat the experimental data obtained for the various substrates but found no evidence of allosteric interactions (not shown). This is consistent with the results reported by Lillehaug and Kleppe (*41*) for dT3′p and the ribonucleotide 3′-monophosphates.

The apparent specificity constant (k_{cat}/K_m) obtained for dG3′p was found to be approximately 4-fold higher than that obtained for dG3′p-C8-4ABP and 20–125-fold higher than those determined for the monoarylamine adducts. This strongly suggests that dG3′p-C8-Ar adducts are intrinsically poorer substrates for PNK than dG3′p. Although the lability of the adducts under alkaline conditions cannot be dismissed, it should be noted that the incubations were conducted for a short time (5 min) and that we did not detect a significant decrease in the concentration of dG3′,5′p-C8-4ABP over a period of 2 h. When it is considered that all the adducts investigated were structurally similar, and that 4-aminobiphenyl is bulkier than single-ring arylamines, the reasons for the better labeling efficiency of dG3′p-C8-4ABP compared to those obtained for monoarylamine adducts are unclear. It is noteworthy that the apparent specificity constants obtained with the alkylaniline-derived adducts exhibited substantial variation with the position of the alkyl substituents, decreasing in the following order: 4MeA > 3,4diMeA > 3MeA > 2,4diMeA. In addition, we obtained very poor and irreproducible labeling with dG3′p-C8-2MeA and dG3′p-C8-2,3diMeA, for which no kinetic parameters could be obtained. Taken together, these observations suggest that arylamine adducts containing an alkyl substitution ortho to the arylamine nitrogen are poorer substrates for PNK than adducts not containing ortho substituents, and that substitution para to the arylamine nitrogen (e.g., 4-aminobiphenyl and 4-methylaniline) is less likely to hamper the labeling process. In previous work (*34*, *35*), we found that monoarylamine adducts containing ortho alkyl substituents have a higher propensity to adopt low-energy syn conformations about the glycosyl bond. Thus, the decrease in the labeling efficiencies observed with the ortho-substituted adducts may be related, in part, to steric factors. In this context, it may be significant that the phenyl rings in 4-aminobiphenyl are not coplanar (*40*, *59*), which may result in a less hindered environment near the active site than would be expected for a bulky aromatic segment.

Conclusions

We have conducted a comparative kinetic study of the PNK-catalyzed ^{32}P-postlabeling of dG3′p and a series of dG3′p-C8-Ar adducts (Ar being 4ABP, 3MeA, 4MeA, 2,4MeA, and 3,4diMeA) from aromatic amines present in tobacco smoke. The results indicate that optimal labeling requires [γ-^{32}P]ATP concentrations substantially higher than those used in typical ^{32}P-postlabeling protocols and confirm that the adducts are labeled with lower efficiencies than dG3′p, with the apparent specificity constants decreasing in the following order: 4ABP > 4MeA > 3,4diMeA > 3MeA > 2,4diMeA. Substitution ortho to the arylamine nitrogen further decreased the labeling efficiency, which may be related, in part, to the higher propensity of ortho-substituted adducts to adopt low-energy syn conformations about the glycosyl bond. These findings suggest that normal nucleotides will be labeled preferentially to dG3′p-C8-Ar adducts under kinetically controlled conditions, which is consistent with the underestimation of arylamine adduct levels by RAL, when compared to measurements of the extent of ^{3}H incorporation (*38*, *39*). If the high specific activity of carrier-free [γ-^{32}P]ATP is taken into account, increasing the ATP concentration in current protocols to achieve optimal labeling does not seem feasible. Therefore, our data emphasize the need for using highly efficient adduct enrichment procedures prior to ^{32}P-postlabeling reactions.

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