[CANCER RESEARCH 50, 3005-3012, May 15, 1990]

Detection of 1,N^6-Propanodeoxyadenosine in Acrolein-modified Polydeoxyadenylic Acid and DNA by ^{32}P Postlabeling[1]

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ABSTRACT

The interaction of acrolein, an α,β-unsaturated aldehyde, with polydeoxyadenylic acid and DNA has been investigated using ^{32}P-postlabeling analysis. In preliminary experiments, polydeoxyadenylic acid was incubated with excess acrolein and then digested to 3′ monophosphates prior to transfer of ^{32}P from [γ-^{32}P]ATP with T4 polynucleotide kinase. The 3′,5′-bisphosphates were 3′-dephosphorylated prior to two-dimensional thin layer chromatography on polyethyleneimine-cellulose layers. Autoradiography provided evidence for the formation of one extra spot of radioactivity, compared to the control. To determine the adduct structure, deoxyadenosine-5′-monophosphate was incubated with a 3-fold excess of acrolein. This material was mixed with a ^{32}P-labeled digest of acrolein-polydeoxyadenylic acid, and the sample was analyzed by ion-pair high performance liquid chromatography. The spot of ^{32}P observed by thin layer chromatography co-eluted with the major product of the acrolein nucleotide reaction mixture, which was purified by ion-pair high performance liquid chromatography. Two-dimensional nuclear magnetic resonance spectroscopy and mass spectrometry showed the adduct to be 3-(2′-deoxyribosyl-5′-monophosphatyl)-7,8,9-trihydro-9-hydroxy-pyrimido[2,3-*i*]purine (1,N^6-propanodeoxyadenosine-5′-monophosphate). High performance liquid chromatography was used to fractionate digests of acrolein-modified DNA prior to detection of this exocyclic adduct by ^{32}P-postlabeling.

INTRODUCTION

Acrolein is the structurally simplest member of the class of α,β-unsaturated aldehydes. In addition to its widespread use in the chemical industry (1), acrolein occurs in cigarette smoke (2) at relatively high concentrations (10–140 μg/cigarette) and is a primary metabolite of cyclophosphamide (3). Cigarette smoking is considered to be a major etiological factor in the induction of bladder cancer in the United States and most Western countries, and approximately one third to one half of all cases in the United States are attributed to this practice (2, 4). Patients given cyclophosphamide therapy who survive for periods longer than 5 years have a greatly increased risk of developing carcinoma of the urinary bladder (5, 6). Direct instillation of acrolein into the bladder lumen of rats induces the same hemorrhagic cystitis and reactive hyperplasia (7–9) seen after i.p. injection of cyclophosphamide. Intraperitoneal injection of acrolein to rats induces urinary bladder hyperplasia (10). Acrolein induces base pair substitution mutations in *Salmonella typhimurium* in the absence of an activating system (11) and reacts directly with DNA (14). It is also mutagenic to fibroblasts from xeroderma pigmentosum patients (12) and Chinese hamster V79 cells (13) but not to normal human fibroblasts (12). Reaction of acrolein with deoxyguanosine has been shown to result in the formation of cyclic 1,N^2-propanodeoxyguanosine adducts, and these products have also been detected in DNA modified by acrolein *in vitro* (14). Reaction of acrolein with deoxycytidine-5′-monophosphate results in the formation of 3,N^4-propanodeoxycytidine-5′-monophosphate (15), but ^{32}P-postlabeling analysis failed to detect a stable nucleotide adduct in polydeoxycytidylic acid that had been incubated with acrolein (16).

The extent to which acrolein modifies other nucleosides in DNA, the structures of the products, and the mechanism(s) for the repair of these adducts or their ability to reduce the fidelity of DNA synthesis have not been investigated. Such knowledge is essential to an understanding of the relevance of different lesions to the carcinogenic process. A ^{32}P-postlabeling procedure (17, 18) has been adapted for the detection of adenine adducts produced by reaction of acrolein with poly(dA)[4] or DNA. This technique involves the HPLC fractionation of deoxynucleoside-3′-monophosphates obtained by digestion of acrolein-modified DNA. The adenine adduct was identified by co-chromatography with marker compounds prepared by reaction of acrolein with deoxyadenosine-5′-monophosphate. Structural characterization of the adduct was accomplished by two-dimensional NMR and mass spectrometry.

MATERIALS AND METHODS

Chemicals. Poly(dA), T4 polynucleotide kinase (wild-type), nuclease P1 (*Penicillium citrinium*), and deoxyadenosine-3′,5′-bisphosphate were obtained from Pharmacia-LKB Biochemicals (Piscataway, NJ). Micrococcal nuclease (*Staphlococcus aureus*) and phosphodiesterase II (bovine spleen) were purchased from Cooper Biomedical (Malvern, PA). [γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham International (Arlington, IL), while nonradioactive ATP was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Deoxyadenosine monophosphates, calf thymus DNA, and acrolein (Gold Label reagent, 99.9% pure, containing 200 ppm hydroquinone) were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI), respectively. PEI-cellulose layers (20 x 20 cm on plastic sheets without fluorescent indicator, 1 x 5 cm with fluorescent indicator; Brinkman Instruments, Westbury, NY) were washed by two successive developments (at right angles to each other) with distilled water onto Whatman no. 1 paper wicks before use. Triethylamine and XAR-5 film were purchased from Eastman Kodak Co. (Rochester, NY). Lightning Plus intensifying screens (DuPont, Wilmington, DE) were used for autoradiography. The YMC/AQ column was purchased from YMC, Inc. (Morris Plains, NJ) and all other columns were obtained from Millipore Waters (Milford, MA). All other materials were obtained from commercial sources.

Received 6/22/89; revised 12/27/89.

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[1] This work was supported by grants from the Nebraska State Department of Health (87-01R), NIH Grants CA44886 and CA36727 from the National Cancer Institute, and NIH Research Grant RR01968. Presented in part at the 80th Annual meeting of the American Association for Cancer Research, San Francisco, May 1989 (15).

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[4] The abbreviations used are: poly(dA), polydeoxyadenylic acid; dA5′P, deoxyadenosine-5′-monophosphate; TLC, thin layer chromatography; PEI, polyethyleneimine, HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; HMQC, heteronuclear multiple-quantum correlation; HMBC, heteronuclear multiple-bond correlation; nOe, nuclear Overhauser enhancement. TEAF, triethylamine formate; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance.

^{32}P-postlabeling of Acrolein-modified Poly(dA) and DNA. Poly(dA) (5 absorbance units) was dissolved in PBS (250 μl) and incubated (37°C, 16 h) with excess acrolein (35 nmol/nmol nucleotide phosphate) or an equivalent volume of PBS. Samples were applied to a column (4 x 0.5 cm) of Sephadex G-100 and eluted with water and fractions (1 ml) containing UV-absorbing material were pooled. Aliquots (160 μl containing 10 nmol of nucleotide phosphate) of control or acrolein-modified poly(dA) were mixed with an equal volume of succinate buffer (40 mM sodium succinate, 20 mM $CaCl_2$, pH 6.0) and digested to 3'-monophosphates by incubation (37°C, 2 h) with 10 μl of a 1:1 mixture of micrococcal release and phosphodiesterase II (200 μg of each/ml of succinate buffer). An aliquot of the digest containing 0.5 nmol of nucleotide phosphate was labeled by transfer (37°C, 3 h) of ^{32}P from [γ-^{32}P]ATP (50 μM, 10–20 Ci/mmol) to the 5'-hydroxyl groups of the 3'-phosphates by T4 polynucleotide kinase (0.25 units/μl) in kinase buffer (40 mM bicine NaOH, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM spermidine, pH 9.3). The 3',5'-bisphosphates were incubated (37°C, 1 h) with 3 units of nuclease P_1 in 0.1 volume of phosphatase buffer (500 mM sodium acetate, 100 mM $MgCl_2$, pH 4.5).

Calf thymus DNA was further purified by phenol extraction (19) and was dissolved in PBS (2 mg/ml) prior to incubation (37°C, 16 h) with acrolein and gel filtration as described above. Fractions containing DNA were pooled and digested to 3'-monophosphates, using 2 μg of each enzyme per μg of DNA, as described above. The digestion of DNA was checked by chromatography of an aliquot (2 μl) on a PEI-cellulose plate (1 x 5 cm), which was developed in 2.5 M ammonium formate. The deoxynucleotide-3'-monophosphates elute at the solvent front, while polymeric material is retained at the origin. The DNA digests were freeze-dried, reconstituted in water (250 μl), and fractionated by HPLC. The eluate corresponding to the retention time of 1,N^6-propanodeoxyadenosine-3'-monophosphate was collected and freeze-dried. The sample was reconstituted 9 times in water (200 μl) and once in 10 μM sodium hydroxide, pH 12.0, between successive freeze dryings. The sample was dissolved in water (80 μl) prior to postlabeling as described above.

Synthesis of Acrolein-modified Nucleotides. Deoxyadenosine-5'-monophosphate was dissolved in PBS (6 mg/ml) and incubated (37°C, 48 h) with a 1- or 3-fold molar excess of acrolein; deoxyadenosine 3',5'-bisphosphate was dissolved in PBS (1 mg/ml) and incubated (37°C, 48 h) with a 3-fold excess of acrolein. These reaction mixtures were used as marker compounds in the assessment of mobile phases for TLC. In addition, the 5'-monophosphate reaction mixtures were used for adduct purification prior to structural analysis by NMR spectroscopy and mass spectrometry. Deoxyadenosine-3'-monophosphate was dissolved in PBS (100 mg/ml) and incubated (37°C, 48 h) with a 3-fold excess of acrolein. The reaction mixture was fractionated by HPLC in order to establish the elution position of the 1,N^6-propano adduct as the 3'-monophosphate.

Thin Layer Chromatography. Aliquots (2 μl) of the ^{32}P-labeled digests (containing 5 μCi ^{32}P) were applied to the bottom right hand corner of the PEI-cellulose plate (20 x 20 cm) without intermediate drying. The plate was developed with solvent A (274 mM NaCl, 16 mM Na_2HPO_4, 4.4 mM KCl, 2.9 mM KH_2PO_4) in the first dimension. The plates were air-dried and rotated 90 degrees clockwise before elution with solvent B (isobutyric acid:ammonia:water, 11:1:3) in the second dimension. Chromatograms were air-dried and wrapped in transparent plastic film prior to detection of normal and adducted nucleotides by screen-intensified autoradiography (ambient temperature, 24 h).

HPLC Techniques. Water (triple-distilled in glass) was filtered through a Millipore Norganic cartridge before use. Mobile phases for ion-pair HPLC were prepared as follows: water or methanol was filtered (0.2 μm) and degassed immediately before addition of triethylamine to a concentration of 10 mM and titration with formic acid (TEAF) to pH 5.0 or 6.0, respectively. Phosphoric acid was used as the counter ion to triethylamine for ion-pair HPLC analysis of ^{32}P-labeled samples. HPLC was performed with a Waters system consisting of two model 510 pumps, a model 680 automated gradient controller, a Waters Intelligent Sample Processor, an oven, and a temperature control module. A model 481 UV/visible detector operated at 254 nm was interfaced with a Hewlett-Packard (Avondale, PA) model 3390A reporting integrator to provide online recording of UV absorbance. A 25 x 0.46 cm (internal diameter) 37 μm silica column was installed between the pumps and the sample processor. Except where otherwise stated, all columns were maintained at 70°C.

Fractionation of 3'-Monophosphates from DNA Digests. A 30- x 0.39-cm (internal diameter) 10-μm Bondapak C_{18} column was eluted at a flow rate of 1 ml/min, at 50°C, with 100 mM ammonium acetate in water, pH 7.0, for 10 min after injection, followed by a linear gradient (curve 6) to methanol:water (50:50), pH 7.0, over 10 min before returning to the initial conditions.

Analysis of ^{32}P-labeled Digests. Two 30- x 0.39-cm (internal diameter) 10-μm Bondapak C_{18} columns were connected in series and eluted at a flow rate of 1.0 ml/min. Starting 2 min after injection, the system was programmed from 0 to 10% solvent B (10 mM TEAF in methanol, pH 6.0) in solvent A (10 mM TEAF in water, pH 5.0), using curve 2 in 10 min, followed by a linear gradient (curve 6) to 30% by 50 min. Thereafter, a linear gradient to 100% solvent B in 3 min was followed by a 3-min isocratic plateau before returning (via curve 6) to solvent A by 60 min. In an alternative system, a 30- x 0.46-cm (internal diameter) 5-μm YMC-AQ C_{18} column was eluted isocratically with 100 mM KH_2PO_4, pH 4.0, at 50°C. ^{32}P-labeled material was detected by a Ramona LS-5 flow-through radioactivity monitor Q (Raytest USA Inc., Pittsburgh, PA) interfaced with an IBM-AT computer. The radioactivity monitor was operated in the stream split mode and was controlled by use of a timed events program.

Adduct Purification. Two 30- x 0.78-cm (internal diameter) 10-μm Bondapak C_{18} columns were eluted as described above, except that the flow rate was increased to 1.5 ml/min. UV-absorbing materials were collected by a FRAC-100 fraction collector (Pharmacia-LKB Biotechnology, Piscataway, NJ) operated in the peak-cutting mode.

Removal of Ion-Pair Reagent by Solvent Extraction. Peaks of UV-absorbing material collected from ion-pair HPLC of the deoxyadenosine-5'-monophosphate acrolein reaction mixture were pooled and adjusted to pH 10.0 by the addition of 0.1 M NaOH. The solution was extracted, in as short a period of time as possible (typically 3–4 min at room temperature), with an equal volume of ether and the pH was returned to 10.0 with 0.1 M NaOH before further extraction with ether. This process was repeated 3 times. The solution was then adjusted to pH 3.0 by addition of 0.1 M HCl and was extracted 3 times with ether. The solution was concentrated by rotary evaporation at 35°C and desalted by chromatography on a column of Sephadex G-25 that was eluted with water.

NMR Spectroscopy. All experiments were performed on a Varian XL-300 spectrometer at 27.0°C, using the standard 5-mm broad-band switchable probe. The two-dimensional experiments were performed non-spinning. In order to perform the indirect detection experiments, the local oscillator frequency was switched from the decoupler transmitter board. This requires elimination of a ground, which prevents use of the local oscillator output in normal detection mode.[5]

RESULTS

Adduct Detection. ^{32}P-labeled digests of control and acrolein-modified poly(dA) were subjected to analysis by two-dimensional TLC; incubation of poly(dA) with acrolein resulted in the formation of an extra spot of radioactivity compared to the control (Fig. 1). This new material had R_f values identical to those of marker compounds prepared by reaction of acrolein with dA5'P (Table 1), and there was no evidence for the formation of adducted bisphosphates. Further analysis by ion-pair HPLC showed that ^{32}P-labeled digests of acrolein-poly(dA) contained two adducts which co-chromatographed with markers synthesized by incubation of acrolein with dA5'P (Figs. 2 and 3). The conversion of dA5'P to adducts was enhanced by increasing the molar ratio of acrolein to dA5'P from 1:1 (Fig. 4*A*) to 3:1 (Fig. 4*B*); indeed adduct 2 was only formed in the

[5] Dr. Steve Palt, Varian Instruments Division (Palo Alto, CA), personal communication.

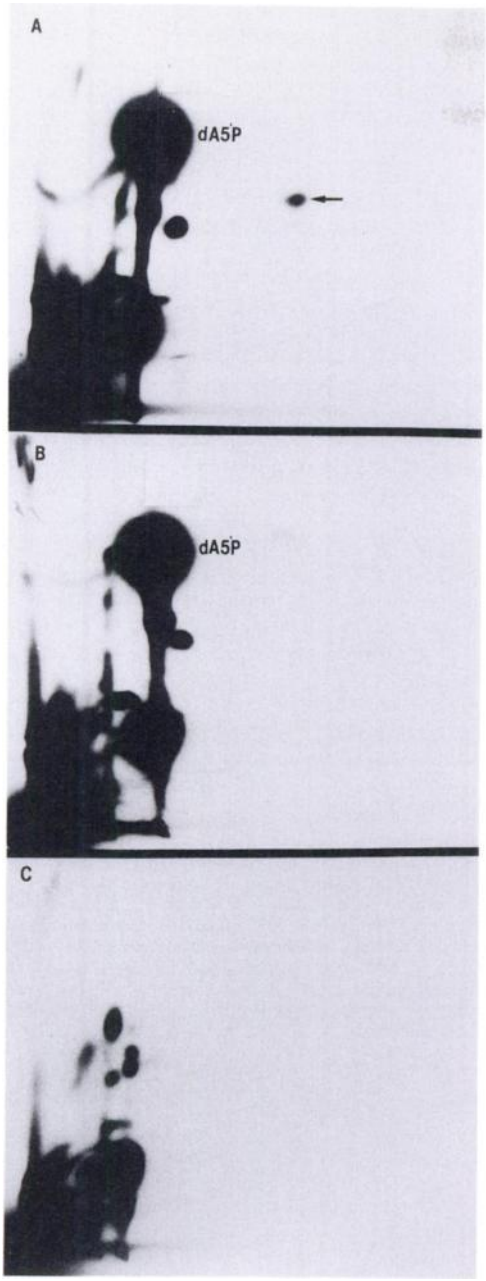

Fig. 1. Detection of an adducted nucleotide in acrolein-modified poly(dA) by ^{32}P-post labeling and TLC. *A*, acrolein/poly(dA); *B*, poly(dA) control; *C*, [γ-^{32}P] ATP control.

Table 1 *R_f values of normal and acrolein-modified deoxynucleotides on PEI-cellulose thin layer chromatograms*

Sample	R_f	
	Solvent A	Solvent B
dA5'P	0.219	0.663
dA5'P/acrolein reaction mixture	0.598	0.610
dA3'5'BP[a]	0.011	0.244
dA3'5'BP/acrolein reaction mixture	0.118	0.244
ATP	0.03	0.03

[a] dA3'5'BP, deoxyadenosine-3',5'-bisphosphate.

presence of excess acrolein (Fig. 4, *B versus A*). An additional early eluting material was formed (peak a) in both reaction mixtures. These adducts were purified by HPLC (Fig. 4), and the ion-pair reagent was removed by solvent extraction prior to structural characterization by NMR and mass spectrometry. Ethereal extraction at pH 10.0 resulted in a 20% loss of adduct

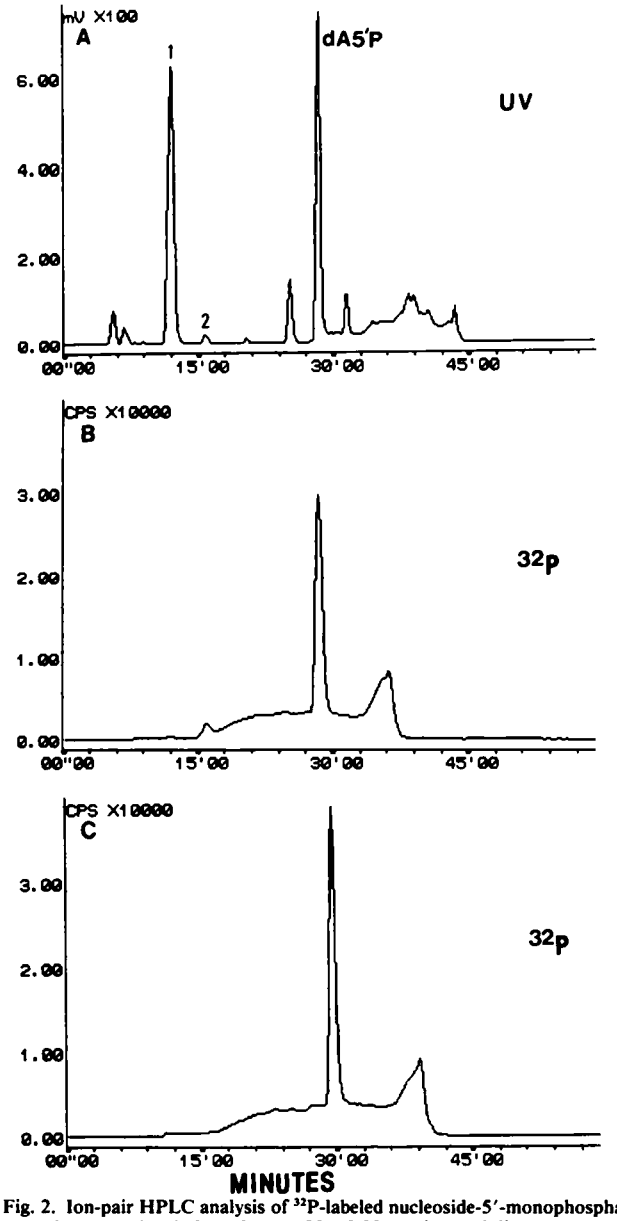

Fig. 2. Ion-pair HPLC analysis of ^{32}P-labeled nucleoside-5'-monophosphates on two 10-μm Bondapak C_{18} columns (30 x 0.39 cm, internal diameter) at 1 ml/min at 70°C. *A*, elution profile of acrolein-modified deoxyadenosine 5'-monophosphate (UV markers); *B*, ^{32}P-postlabeling analysis of acrolein-modified poly(dA); *C*, ^{32}P-postlabeling analysis of poly(dA). Peak identification: peak 1, adduct 1 (1,N^6-propanodeoxyadenosine-5'-monophosphate); peak 2, adduct 2 (presumed to be an unstable diadduct). Saturation of the radioactivity monitor by excess [γ-^{32}P]ATP was prevented by use of a timed events program to activate a stream splitter (set at 100%) and divert the HPLC eluate to waste while purging the flow cell at 5 ml/min with 1.0 M phosphoric acid. Inactivation of the stream splitter and pump diverted the HPLC eluate into the flow cell. This sequence of events also eliminates the peak of inorganic ^{32}P from the beginning of the chromatogram and accounts for the changes in ^{32}P baseline seen at 10 and 40 min (*C*). In *B*, the stream split was inactivated at an earlier time (7 min) and this accounts for the baseline fluctuation between 7 and 10 min.

1 (based on UV absorbance). A smaller loss (5%) was noted during extraction at pH 3.0. These changes in pH did not alter the chromatographic properties of adduct 1; neither did they result in the formation of new products when the adduct solutions were reanalyzed by ion-pair HPLC. The ^{1}H NMR spectrum of adduct 1 was unchanged following extraction.

Preliminary experiments to detect acrolein-adenine adducts by ^{32}P-labeling of total DNA were unsuccessful, and an alternative procedure was developed, using HPLC fractionation of deoxyadenosine-3'-monophosphate/acrolein reaction mixtures to establish the retention time of the 1,N^6-propano adduct. The

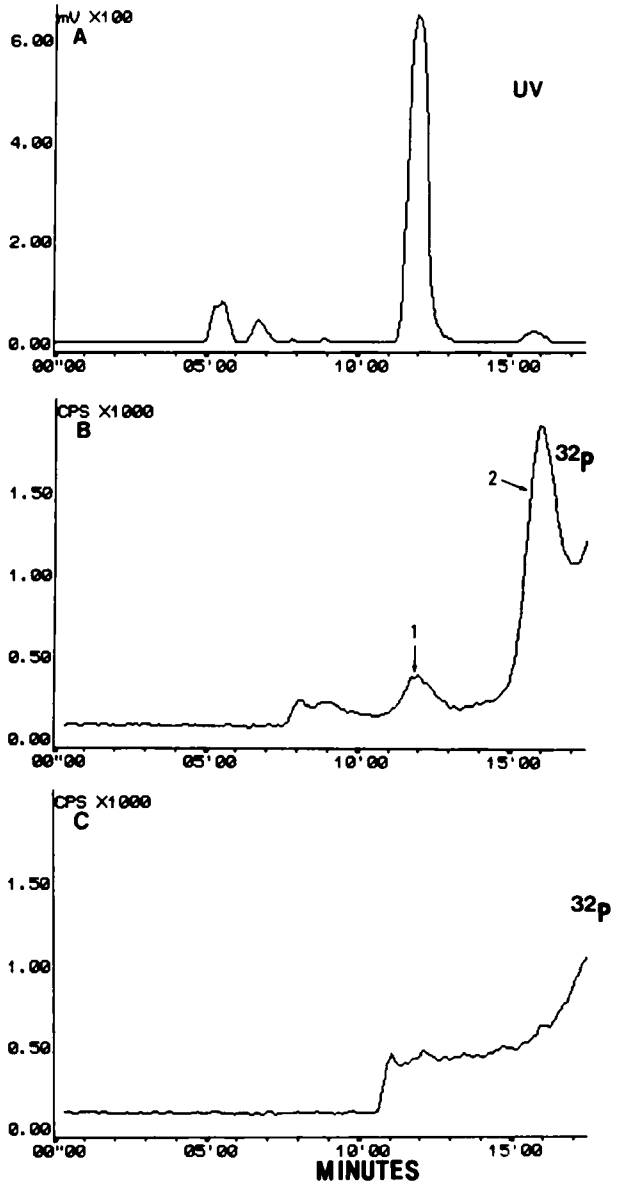

Fig. 3. Scale expansion of Fig. 2, showing elution position of the UV marker compounds (*A*), adducts 1 and 2 in acrolein-modified poly(dA) (*B*), and the poly(dA) control (*C*). The rise in ^{32}P baseline at 10 min (*C*) is due to diversion of radioactive eluate into the flow cell after a phosphoric acid purge. In *B*, the stream split was inactivated at an earlier time (7 min) and this accounts for the baseline fluctuation between 7 and 10 min.

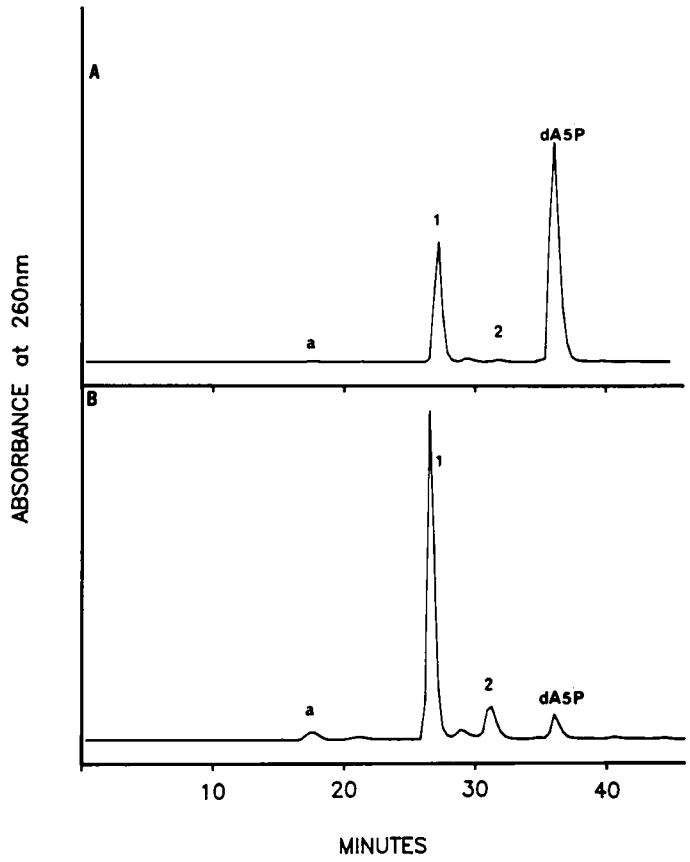

Fig. 4. Ion-pair HPLC purification of acrolein deoxyadenosine-5′-monophosphate reaction mixture on two 10-μm Bondapak C_{18} columns (30 x 0.79 cm, internal diameter) at 1.5 ml/min at 70°C. *A*, equimolar amounts of each reactant; *B*, 3-fold excess of acrolein. In each case, an aliquot of the reaction mixture corresponding to 3 μmol of deoxyadenosine-5′-monophosphate was analyzed. Peak identification: peak a, acrolein adduct of adenine (free base); peak 1, adduct 1 (1,N^6-propanodeoxyadenosine-5′-monophosphate); peak 2, adduct 2 (presumed to be an unstable diadduct).

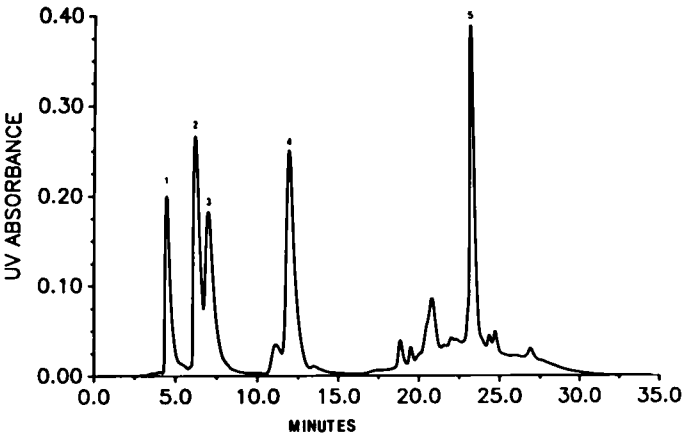

Fig. 5. HPLC analysis of deoxyadenosine-3′-monophosphate-acrolein reaction mixture. Peak 2, deoxyadenosine-3′-monophosphate; peak 3, 1,N^6-propanodeoxyadenosine-3′-monophosphate.

peaks of UV-absorbing material were collected, freeze-dried, and postlabeled as described above. Analysis of the ^{32}P-labeled fractions showed that peak 3 (Fig. 5) provided a radioactive product which co-chromatographed with 1,N^6-propandeoxyadenosine-5′-monophosphate in TLC (with solvent A, data not shown) or isocratic HPLC on the YMC/AQ C_{18} column. For analysis of acrolein-modified DNA, the eluate corresponding to the retention time of peak 3 was collected and postlabeled prior to HPLC analysis (Fig. 6).

Structural Determinations by NMR and Mass Spectrometry. Structural assignment of the adduct began by obtaining the ^1H and ^{13}C NMR spectra shown in Fig. 7. The structure of the adduct is shown in Fig. 7, *inset*. The assignments of individual protons, except for the aromatics, was accomplished by a two-dimensional correlation spectroscopy experiment (20). The contour plot of that experiment is shown in Fig. 8. The off-diagonal resonances indicate scalar coupling between the correlated protons; hence, the two-dimensional correlation spectroscopy experiment allows a stepwise assignment of the scalar coupling networks. The resonances of the deoxyribose moiety are easily recognized, and the assignments are made starting with the anomeric H1′ proton. The remaining AA′MPX system represents the exocyclic propano moiety, with the most downfield resonance in the system representing the single proton on C9. The aromatic resonances H2 and H5 have no coupling partners and therefore, could not be assigned unambiguously by this experiment. For this reason, they are not shown. These

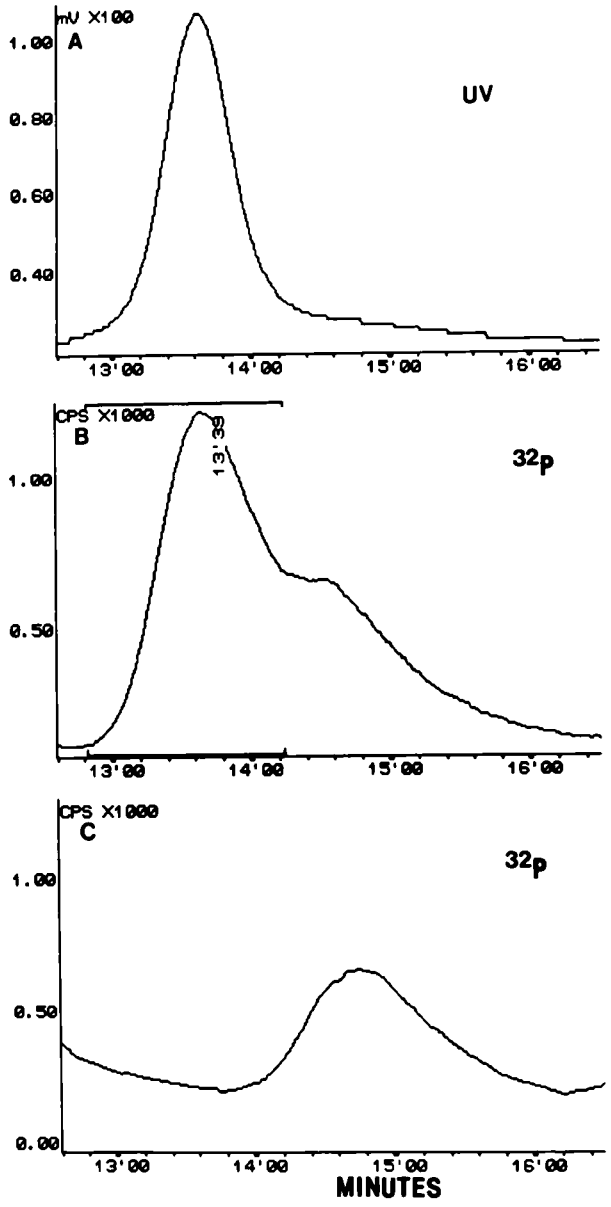

Fig. 6. Detection of 1,N^6-propanodeoxyadenosine-5′-monophosphate in an HPLC-purified digest of acrolein-modified DNA (*B*) or control DNA (*C*). Only the region corresponding to the elution position of the purified adduct is shown (*A*). [γ-^{32}P]ATP elutes at 6 min in this isocratic HPLC separation and a timed events program was used to purge the flow cell and divert eluate to waste until 11 min after injection.

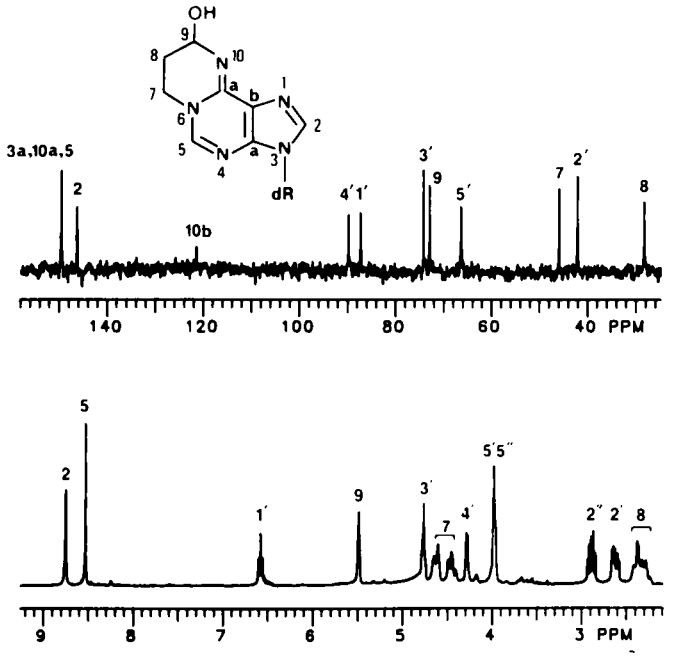

Fig. 7. One-dimensional proton spectrum and carbon spectrum of adduct 1. The solvent is D_2O and the spectra were acquired at 27°C.

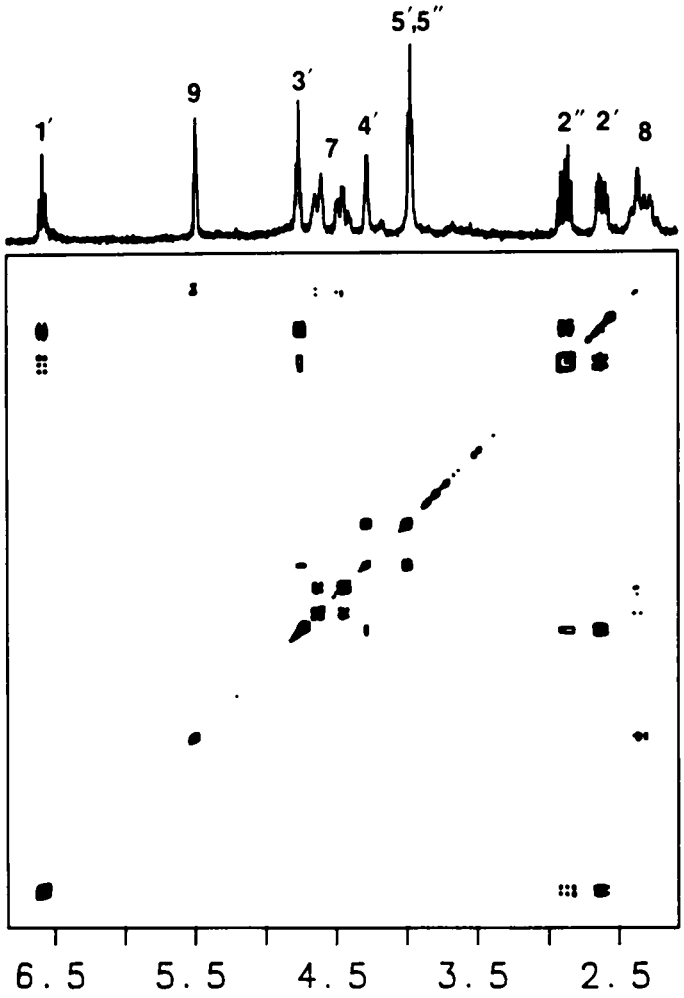

Fig. 8. Two-dimensional correlation spectrum of adduct 1.

protons are readily assigned by correlation to ^{13}C resonances in the second of the two experiments described below.

The HMQC experiment (21) is a very sensitive means of assigning ^{13}C resonances through their correlation with assigned ^{1}H signals. The result of such an experiment is shown in Fig. 9. The normal ^{1}H NMR spectrum is shown along the left vertical axis of the HMQC data. The ^{13}C spectrum of the adduct is shown along the top. The contours in the two-dimensional plot connect protons with the carbon to which they are directly bound. All carbons from the sugar, as well as from the exocyclic propano group, are easily assigned. Assignment of the aromatic protons, however, requires HMBC (22), which is a long-range version of the HMQC experiment.

The HMBC experiment, like HMQC, correlates protons with carbons via scalar coupling. The difference is that, rather than correlating protons only to their directly attached ^{13}C, it allows protons to be correlated to carbons which are at least 2 (or more) bonds away. Fig. 10 shows a portion of the HMBC experiment performed on the adduct. The anomeric proton is shown along the vertical axis, and a portion of the ^{13}C spectrum

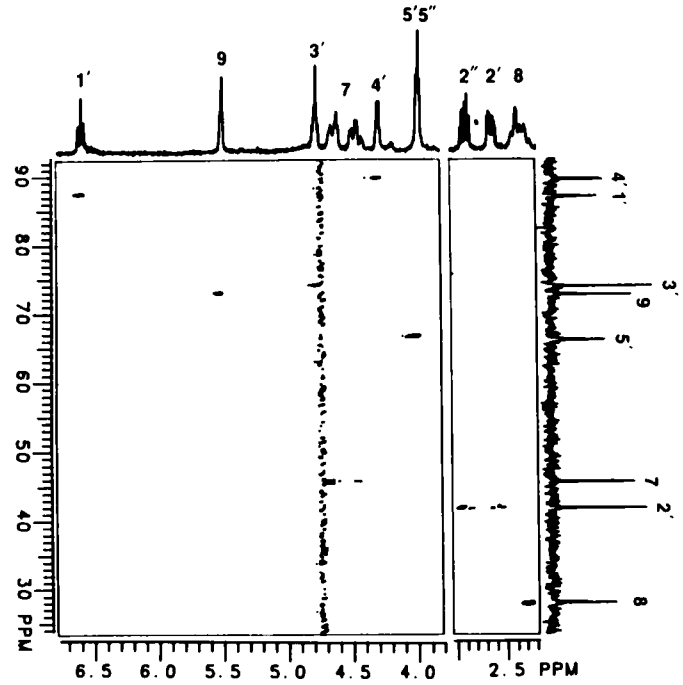

Fig. 9. HMQC spectrum of adduct 1. The standard proton and carbon spectra are shown along the *vertical* and *horizontal axes*, respectively. Note that the correlation of H2 and H5 to their carbons is not shown, because unambiguous assignment requires the HMBC experiment.

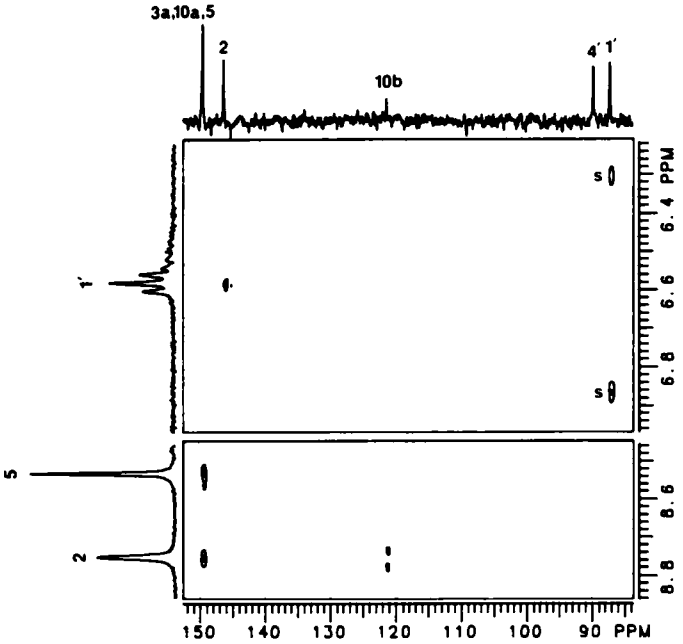

Fig. 10. HMBC spectrum of adduct 1. *s*, the one-bond coupling satellites of H1'. All other correlations are over multiple bonds.

is above the contour plot of the HMBC spectrum. The two contours labeled *s* are the ^{13}C satellites of the anomeric proton. These satellites correlate the anomeric proton to its directly attached carbon at 87.27 ppm, in agreement with the HMQC assignment. In addition, there is a single long-range correlation of the same proton to the upfield aromatic carbon. This represents long-range scalar coupling between the anomeric proton and one of the protonated aromatic carbons. This must represent the C2 carbon, since correlation of the H1' proton to the C5 would require observable scalar coupling over 5 bonds, which is not possible at this resolution. This single piece of information allows unambiguous assignment of the aromatic protons, because C5 may be assigned by exclusion to be the carbon at 149.45 ppm. Interestingly, the $C3_a$ and $C10_a$ carbons nearly perfectly overlap the C5 at the resolution of this experiment. As can be seen, H2 is correlated to two quaternary carbons, one at 149.65 ppm and one at 121.15 ppm. Because H5 also shows a long-range correlation to the carbon at 149.65 ppm, this must represent $C3_a$, since it is the only quaternary carbon within 3 bonds of both H2 and H5. By exclusion, the other quaternary at 121.15 ppm may be assigned as $C10_b$, due to its proximity to H2. On close inspection, H5 also correlates to a carbon at 149.55 ppm, which may then be assigned as $C10_a$, which would be expected to show coupling to H5. The resolution in the figure is inadequate for visual detection of this.

The only remaining problem was assignment of orientation of the exocyclic propano function. It was hoped that assignment could be made via correlations in the HMBC experiment. However, the coupling was too small to give a detectable correlation, and an nOe difference experiment was used instead. This technique allows correlation of protons which are close in space (<5Å), by observation of an enhancement in the intensity of one proton during irradiation of another. The results of such an experiment are shown in Fig. 11. A significant nOe was observed at the H5 proton when the upfield H7 adduct proton was irradiated, which shows these two protons are less than 5Å apart in space. The intensity of the nOe was dependent upon irradiation time, indicating that the observed enhancement was not an experimental artifact. No nOe was observed in any proton when H9 was irradiated, as would be expected from the structure of the adduct.

Fast atom bombardment mass spectrometry of the ammonium salt of the adduct results in an $(M+H)^+$ species with an experimentally determined mass of 388.1031 units. This is consistent with an empirical formula of $C_{13}H_{19}N_5O_7P$ (theoretical, 388.1022; relative deviation, 2.3 ppm). MS/MS can provide additional structural information in the study of nucleosides. In the MS/MS of protonated nucleosides, the most facile fragmentation is of the *N*-glycosyl bond to form the protonated

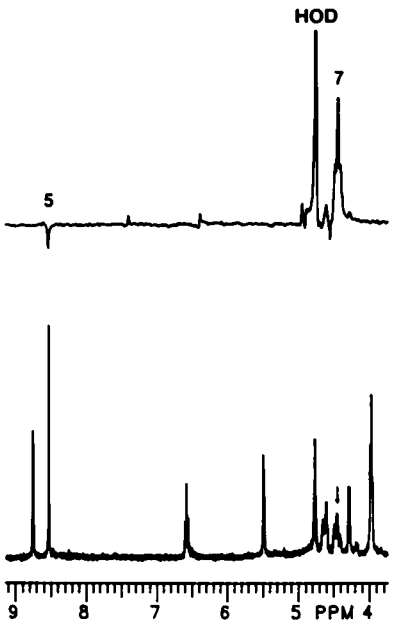

Fig. 11. nOe difference experiment shown above the standard proton spectrum of adduct 1. The irradiation time used was 3.0 s for saturation of the upfield H7 resonance.

nucleoside base BH_2^+ (23). For modified nucleosides, this provides information to determine if the chemical modification is in the base or in the sugar moiety. If the mass of the BH_2^+ ion in the MS/MS spectrum is that of the normal base, the modification must be located within the sugar unit. If, however, the BH_2^+ ion is of higher mass, the modification involves the base. MS/MS of the $(M+H)^+$ species results in an abundant fragment ion of m/z 192 (not of m/z 136, BH_2^+ for adenine). This confirms that the acrolein modification resides in the base.

DISCUSSION

^{32}P-postlabeling was used to detect nucleotide adducts in poly(dA) and DNA that had been incubated with excess acrolein. The adducted nucleotides were sensitive to 3′-phosphorylation by nuclease P_1, since they chromatographed (in three systems) with markers synthesized by reaction of dA5′P with acrolein. These adducts were purified from the marker mixture by ion-pair HPLC, and the ion-pair reagent was removed by solvent extraction prior to the structural analysis. NMR spectroscopy showed adduct 1 to be 1,N^6-propanodeoxyadenosine-5′-monophosphate. This product was formed by Michael addition of N1 of adenine to the C3 of acrolein and subsequent ring closure between the exocyclic amino group at the 6-position of adenine and C1 of acrolein. Mass spectral data for adduct 1 were also consistent with mono-adduction of the adenine base by acrolein. The orientation for the addition was unequivocally established by the demonstration of a spatial interaction (nOe) between the methylene protons of the C3 from acrolein and the H2 of the adenine base. This provides proof for the structural assignment shown (Fig. 7), since the nOe would not be detected if the alternative orientation were to occur. A similar adduct formed by reaction of acrolein with deoxycytidine-5′-monophosphate has also been identified (15). These structural assignments are the reverse of those reported previously, where oxidation of the acrolein adduct of 9-methyladenine yielded a product characterized by its spectral properties. On this basis and by analogy with the structure inferred by oxidation of the acrolein adduct of 1-methylcytosine, the alternative assignment for the orientation of addition was made (24, 25).

Efforts to characterize adduct 2 were unsuccessful, since this material converts into adduct 1 upon isolation. The available NMR data (not shown) suggest a disubstituted moiety which only forms in the presence of excess acrolein. A similarly unstable diadduct was also observed upon modification of deoxycytidine-5′-monophosphate (15), and multiple adduction of purine nucleosides has been reported following reaction with acrolein (25) or malondialdehyde (26, 27). Exocyclic adducts of guanine have been detected upon reaction of deoxyguanosine or DNA with acrolein (12). This latter study used a fluorescence assay for the detection of guanine adducts released by mild acid hydrolysis of DNA modified by acrolein *in vitro*. The products were shown to be a mixture of interconverting diastereomers (adducts 1 and 2), while adduct 3 was presumed to be a mixture of diastereomers and showed no circular dichroism spectrum, in agreement with this assumption. The 1,N^6-propanodeoxyadenosine adduct also contains a new chiral center at C9, but there was no evidence for diastereomers. Attempts to identify the early eluting material (peak a) from the acrolein-dA5′P reaction mixture by NMR showed it was devoid of deoxyribose protons. In addition, adduct 1 was converted into the adducted base (peak 1) by mild acid hydrolysis (0.1 M perchloroacetic acid, 70°C, 30 min). These data suggested the material was an adducted adenine base produced by scission of the *N*-glycosyl bond.

The amount of acrolein used in this study [35 nmol acrolein/nmol of nucleotide phosphate in poly(dA) or DNA] is identical to that employed in a previous study (14) and is much higher than the amount used in biological tests such as mutagenicity assays (11, 12, 13). The data presented show that ^{32}P-postlabeling can be applied to the detection of an acrolein adduct in DNA modified *in vitro* and that this sensitive technique may be of value in studying the persistence of these exocyclic adducts in cellular DNA. Acrolein induces base pair substitution mutations in *Salmonella* TA104 without metabolic activation (11). The mutational site is the nonsense sequence TAA, and it seems probable that the 1,N^6-propanodeoxyadenosine adduct reported here may contribute to the biological effect(s) of acrolein in this mutation assay. The involvement of the N1 and exocyclic amino group of adenine in Watson-Crick base pairing and its disruption by adduction with acrolein substantiates this hypothesis.

ACKNOWLEDGMENTS

We thank Dr. Donald L. Nagel for assistance with NMR studies and Ilene Pinnt, Russell Lidberg, and T. Scott Tibbels for technical assistance. The secretarial support of Jan Leemkuil and Deboraha Coleman are gratefully acknowledged.

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