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Reactivity and adduct formation of a polyaromatic hydrocarbon, 7-bromomethylbenz[*a*]anthracene, with chromatin histone proteins

PAMELA C. STACKS*

Department of Chemistry, San Jose State University, San Jose, CA 95192 (U S A)

and

JOSEPH A. MAZRIMAS, MICHELE CORZETT and ROD BALHORN

Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94550 (U S A.)

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SUMMARY

The alkylation of histones by the direct-acting carcinogen 7-bromomethylbenz[*a*]anthracene was demonstrated both in vivo and in vitro. The relative molar reactivity for mouse liver histones in vivo was H3 > H1 > H2b > H4 > H2a. The in vitro modification of histone H3 was examined in detail. Amino acid adducts stable to acid hydrolysis were separated after acetylation by reversed-phase high-performance liquid chromatography and characterized using ultraviolet absorbance spectra and synthetic amino acid adduct standards. Three major adducts were observed and tentatively identified as cysteinyl, lysyl and histidinyl adducts of histone H3.

INTRODUCTION

Carcinogens can have a number of macromolecular target sites owing to their chemical nature and reactivity. Although it is thought that the primary initiation lesions are associated with DNA, it has become clear that chromosomal proteins may be modified as well [1–3]. Our approach has been to examine the formation of adducts to the histones, proteins intimately associated with DNA. Since many DNA lesions are rapidly repaired, determination of the extent of histone modification may prove to be a more accurate biological dosimeter [3].

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Additionally, a comparison of the in vivo and in vitro reactivity of the histones can be used to distinguish between intrinsic chemical reactivity and the accessibility of macromolecular domains in native chromatin structure.

Accordingly, we have used a direct-acting polyaromatic hydrocarbon, 7-bromomethylbenz[*a*]anthracene, which forms well defined DNA adducts [4], to determine the in vivo relative molar reactivity of mouse liver histones. To characterize the extent and site(s) of adduct formation, histone H3 was treated in vitro with the carcinogen, acid-hydrolyzed, and acetylated with acetic anhydride. The acetylated amino acid adduct products were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using UV absorbance spectra and by comparison with N-acetylated amino acid adduct standards. The use of N-acetylated amino acids enabled us to prevent the reaction of the α-amino group with the carcinogen and provided a sharper chromatographic separation of the derivatives.

EXPERIMENTAL

Chemicals

The N-acetylated amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Labeled and unlabeled 7-bromomethylbenz[*a*]anthracene were prepared by bromomethylation of benz[*a*]anthracene (Eastman Kodak, Rochester, NY, U.S.A.) [5]. Tritium-labeled benz[*a*]anthracene was prepared by catalytic exchange by Amersham Radiochemical Centre and was purified in our laboratory by recrystallization from acetone–water solutions. The purity as determined by HPLC was ca. 97%. Crystals were stored at $-20\,^{\circ}$C. All handling of the benz[*a*]anthracene derivatives was done under yellow lighting, and samples were stored in foil. Milligram amounts of 7-bromomethylbenz[*a*]anthracene were weighed out in a glove box.

Apparatus

All chromatographic separations were accomplished on a Beckman gradient elution system, which consisted of two Model 110A solvent-delivery pumps with a solvent programmer. The effluent was passed through a Hewlett-Packard 1040A HPLC diode array detector system, which can monitor variable wavelengths and store spectra taken during the elution. Various columns were used for both purification of the histones and analysis of the amino acid adducts.

Methods

In vivo labeling with 7-bromomethylbenz[a]anthracene. Male mice, strain C57 BL/6, ten to twelve weeks of age (Simonson Labs., Gilroy, CA, U.S.A.) were injected intraperitoneally with 7.7 nmol [^{3}H]7-bromomethylbenz[*a*]anthracene (0.8 Ci/mmol) in 100 μl of dimethylsulfoxide per kg body weight. After 2 h the livers were excised and the nuclei isolated [3].

Specific activity of in vivo labeled histones. Crude nuclei preparations were suspended directly in 3 *M* guanidine hydrochloride, sonicated and briefly centrifuged to remove insoluble material. The chromosomal proteins were separated from DNA by gel permeation chromatography on three TSK-3000 SW columns connected in tandem [3]. The resulting chromosomal proteins were fractionated by reversed-phase chromatography. Each sample was extracted three times with three volumes of buffer-saturated ethyl acetate (saturated with an equal volume of 0.1 *M* sodium borate, pH 9), and the extracted aqueous phase was exposed to nitrogen gas to drive off residual ethyl acetate prior to quantification by scintillation counting. The proteins were quantified using absorbance measurements at 230 nm and an extinction coefficient of 3.31 cm^2/mg.

Histone isolation for in vitro reactions with 7-bromomethylbenz[a]anthracene. A crude nuclear pellet was obtained from untreated mouse livers [3]. The washed nuclei pellet was resuspended by homogenization in 0.25 μM Tris (pH 8) and 0.25 μM EDTA and sonicated to fragment the DNA. The solution was made 0.2 *M* H_2SO_4 and was stirred overnight at 4°C to precipitate nucleic acids. Following centrifugation at 16 490 *g* for 5 min, the supernatant was dialyzed against six volumes of 80% ethanol overnight at 4°C. The precipitated histones were collected by centrifugation (10 550 *g* for 10 min). Isolation of specific histones was achieved by reversed-phase chromatography [3]. The histone pellet in aqueous trifluoroacetic acid (0.1%) was injected into a 10-μm PRP-1 column (two 300 mm×7.5 mm I.D. columns connected in tandem; Hamilton, Reno, NV, U.S.A.) and separation was accomplished by using a multi-step acetonitrile gradient [3]. The purified fractions were characterized by acid–urea polyacrylamide gel electrophoresis [6].

In vitro reactions of 7-bromomethylbenz[a]anthracene and histones. Purified histone H3 (1 mg/ml) was reduced in 10 m*M* dithiothreitol, 0.1 *M* sodium borate (pH 9.2) for 30 min at room temperature. The H3 was precipitated by addition of one fourth volume of 50% (w/w) trichloroacetic acid, kept on ice for 30 min and centrifuged (8000 *g* for 5 min). The resulting pellet was washed three times with acidified acetone and centrifuged at 8000 *g* for 3 min. The pellet was dried under nitrogen gas and subsequently dissolved in 0.1 *M* sodium borate (pH 7.6) at a concentration of 1 mg/ml. The 7-bromomethylbenz[*a*]anthracene was dissolved immediately before use in dimethyl sulfoxide. The final concentrations of the reactants were 130 μg/ml histone H3, 66 μg/ml 7-bromomethylbenz[*a*]anthracene, 33 m*M* sodium borate and 66% dimethyl sulfoxide. The dimethyl sulfoxide was necessary for carcinogen solubility. The samples were incubated for 1 h at room temperature, followed by ethyl acetate extraction (three volumes of ethyl acetate, extracted three times), precipitation with trichloroacetic acid and washing with acidified acetone (three times). The pellet was resuspended in 6 *M* hydrochloric acid for acid hydrolysis (20–24 h at 110°C) at a protein concentration of 0.5–1 mg/ml. The acid hy-

drolysate was lyophilized and resuspended with half-saturated sodium acetate to yield 1 mg/ml. Acetylation of the products was accomplished by addition of a 1/50 volume of acetic anhydride every 10 min for a total of five additions at room temperature [7], followed by lyophilization.

In vitro reaction of 7-bromomethylbenz[a]anthracene and N-acetylated amino acids. Stock solutions of 0.2 *M* N-acetylated amino acids in 0.1 *M* sodium borate (final pH adjusted to 7.8–8.0 by addition of sodium hydroxide or hydrochloric acid) and 1 mg/ml 7-bromomethylbenz[*a*]anthracene were prepared immediately prior to use. The final in vitro reaction mixtures contained 57 m*M* N-acetylated amino acid, 1.2 m*M* 7-bromomethylbenz[*a*]anthracene, 70% dimethyl sulfoxide and 30 m*M* sodium borate (50-fold molar excess of amino acid to carcinogen).

Separation of N-acetylated amino acid methylbenz[a]anthracene adducts. Samples were dissolved in 70% dimethyl sulfoxide and 30 m*M* sodium borate. Separations were accomplished on a Nucleosil 5-μm C_{18} reversed-phase column (300 mm × 7.5 mm I.D. column, Hamilton) using a multi-step methanol gradient. Solvent A contained 20 m*M* sodium acetate (pH 5.9) and 2.5% tetrahydrofuran. Solvent B contained 90% methanol. The gradient consisted of 50% solvent B from 0 to 15 min, a linear gradient of 50 to 100% solvent B from 15 to 40 min and 100% solvent B from 40 to 60 min.

RESULTS

In vivo reactivity of 7-bromomethylbenz[a]anthracene

We have reported previously that the formation of 7-bromomethylbenz[*a*]anthracene adducts to DNA in vivo was at least twenty-fold higher (per nucleotide) than adducts formed to one of the chromosomal proteins, histone H3 (per amino acid residue) [3]. The other histones are also accessible for adduct formation and we have determined the relative molar reactivities of each histone to 7-bromomethylbenz[*a*]anthracene (Table I). H3, the most reactive histone, had ca. fifteen adducts formed per 10^6 molecules, whereas H4 and H2A were the least reactive with ca. six adducts formed per 10^6 molecules. The non-core histone, H1, was the most highly modified with ca. seventeen adducts per 10^6 molecules. However, H1, the largest histone with ca. 200 residues, was less substituted than H3 (135 residues) on an adduct/amino acid residue basis. Although the H1 fraction is enriched for H1 (90%), small amounts of other peptides are present. Therefore, the observed level of H1 modification must represent an upper limit. The high mobility group, basic chromosomal proteins, which eluted first during the reversed-phase chromatography of the histone extracts, were modified to an intermediate extent (when considered as a group) in comparison to the histone proteins.

TABLE I

IN VIVO MODIFICATION OF MOUSE LIVER CHROMOSOMAL PROTEINS BY 7-BROMOMETHYLBENZ[*a*]ANTHRACENE

High-mobility-group proteins are designated as HMG. Mouse liver proteins were labeled in vivo, extracted and fractionated by reversed-phase chromatography [3]. The adducts/molecule were calculated using the specific activity of the [^{3}H]7-bromomethylbenz[*a*]anthracene (0.8 mCi/mmol), determinations of radioactivity associated with isolated proteins and protein contents determined by absorbance at 230 nm.

Protein	Adducts/molecule ($\times 10^6$)
HMG	9.3 ± 1.4
H1	17.3 ± 2.1
H3	14.7 ± 3 3
H2B	11.2 ± 6.4
H4	6.6 ± 1.8
H2A	5.7 ± 1.1

In vitro modification of histone H3 by 7-bromomethylbenz[a]anthracene

We examined the modification of histone H3 in greater detail by characterizing which amino acid adducts form in vitro. The reaction conditions were chosen such that the extent of modification was less than one substitution per histone on average. Acid hydrolysates of 7-bromomethylbenz[*a*]anthracene-modified histone H3 were separated before and after acetylation by reversed-phase chromatography (Fig. 1). Both acetylated and unacetylated amino acids eluted early from the column as a broad peak (t_R = 2–4.5 min) and are completely separated from the acetylated or unacetylated methylbenz[*a*]anthracene-modified amino acids with the gradient used (Fig. 1A and B). Derivatization of the amino acid adducts by acetylation resulted in the resolution of one broad adduct peak (peak 1 in Fig. 1A) into three sharper adduct peaks (Fig. 1B). Methylbenz[*a*]anthracene containing derivatives were easily identified using the characteristic absorbance spectrum of benz[*a*]anthracene (double maxima at 280 and 290 nm) (Fig. 2). Derivatives maintain the double maxima but the absorbance peaks are often shifted by 1–2 nm. In Fig. 1B, only peaks 1, 2 and 3 contain such a spectrum. There is a distinct absence of the benz[*a*]anthracene spectrum associated with any of the other peaks.

The major product of the in vitro reaction in the absence of added protein or amino acids elutes with a retention time of 43 min. 7-Hydroxymethylbenz[*a*]anthracene (an expected product under aqueous conditions) also elutes with a retention time of 43 min. Several minor products elute even later. Thus the peaks designated as amino acid adducts (Fig. 1) were not formed by the reaction of the 7-bromomethylbenz[*a*]anthracene with non-protein components of the in vitro reaction mixture.

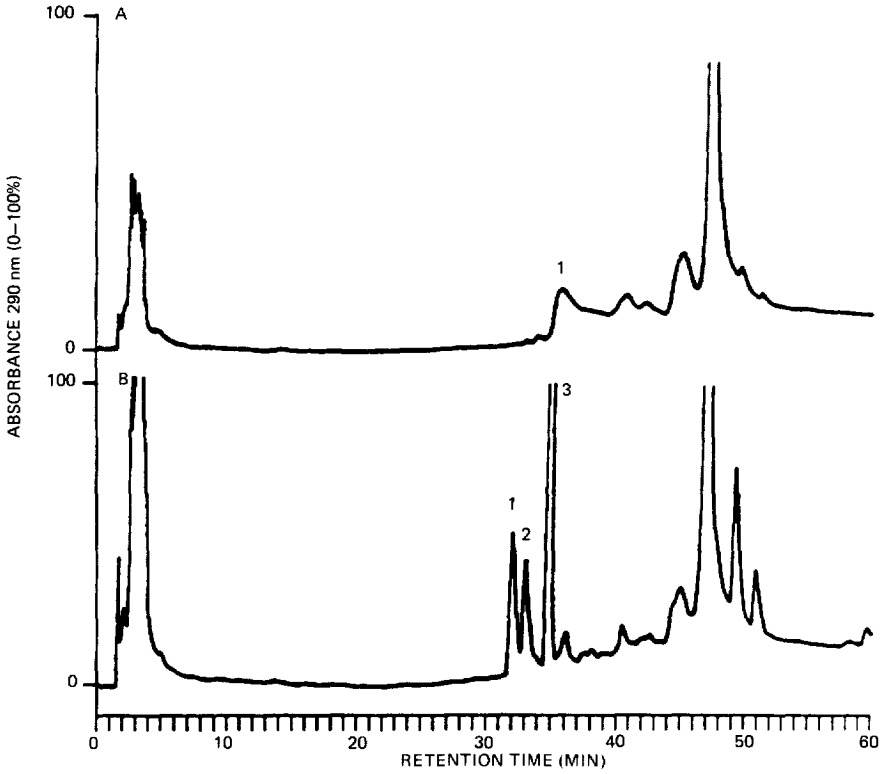

Fig. 1. Reversed-phase HPLC elution profiles of amino acid hydrolysates of 7-bromomethylbenz[*a*]anthracene-treated histone H3 (A) unacetylated and (B) acetylated prior to chromatography. A Nucleosil 5-μm, C_{18} column (300 mm×7.5 mm I.D.) and a multi-step methanol gradient were used. Solvent A, 20 m*M* sodium acetate (pH 5.9) and 2.5% tetrahydrofuran; solvent B, 90% methanol; gradient, 50% solvent B from 0 to 15 min, a linear gradient of 50 to 100% solvent B from 15 to 40 min and 100% solvent B from 40 to 60 min. Absorbance at 290 nm was monitored with the 100% scale equal to 0.025 absorbance units.

In vitro reaction of 7-bromomethylbenz[a]anthracene with N-acetylated amino acids

Various N-acetylated amino acid adducts were derivatized with 7-bromomethylbenzene[*a*]anthracene and the reaction products characterized by reversed-phase chromatography. The retention time of each product was determined and the extent of adduct formation was quantified by integrating peak areas. N-Acetylcysteine was the most reactive amino acid (ca. 70% of the 7-bromomethylbenz[*a*]anthracene formed an adduct), followed by N-acetylmethionine (27%) and N-acetyltyrosine (18%); N-acetylhistidine, N-acetylserine and N-acetyllysine all yielded ca. 10%. N-Acetylarginine was only nominally reactive (ca. 2%). Based on the retention times of these synthetic standards, several of the histone adducts were identified as possible products

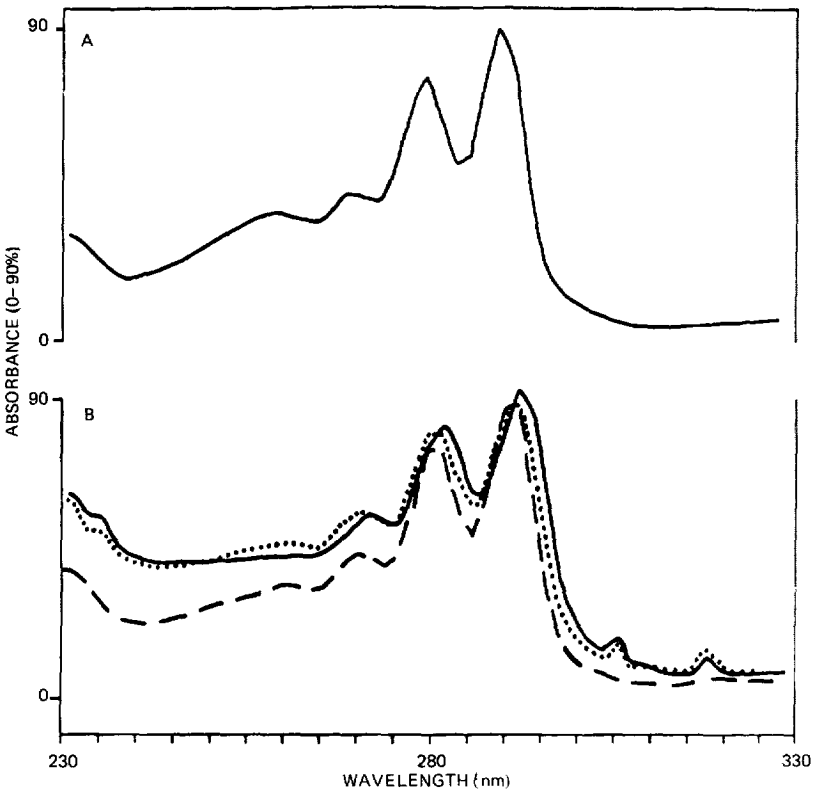

Fig. 2. Characterization of reversed-phase chromatographic peaks by UV spectral analysis: (A) 7-hydroxymethylbenz[*a*]anthracene; (B) peak 1 (solid line), peak 2 (dashed line) and peak 3 (broken line) from Fig. 1B, the acetylated hydrolysate of 7-bromomethylbenz[*a*]anthracene-treated histone H3. The 100% scale is equal to (A) 0.667, (B) peak 1 as 0.011, peak 2 as 0.010 and peak 3 as 0.065 absorbance units.

of the in vitro modification reactions. Acetylated H3 adducts were coinjected with synthetic N-acetylated amino acid adducts to determine whether specific adduct peaks coeluted. Owing to the absence of the hydrolysis product of 7-bromomethylbenz[*a*]anthracene (eluting at 43 min) in the histone elution profile, it is possible to use the area of the peak at 43 min to normalize the concentrations of the coinjections. The coelution patterns (Fig. 3) made it possible to assign probable identities to the H3 adduct peaks as follows: peak 2 (8%) as a lysyl adduct and peak 3, the predominant adduct in H3 (58%), as a cysteinyl adduct. Peak 1 (10%) was tentatively identified as a histidinyl adduct, but this identification is less certain owing to the more complex N-acetylhistidine adduct standard elution profile.

Another possible location for adduct formation in histone H3 is the α-amino group of the N-terminal amino acid, alanine [8]. Although alanine is quite reactive (30% as an isolated amino acid), its adduct coelutes as a leading shoul-

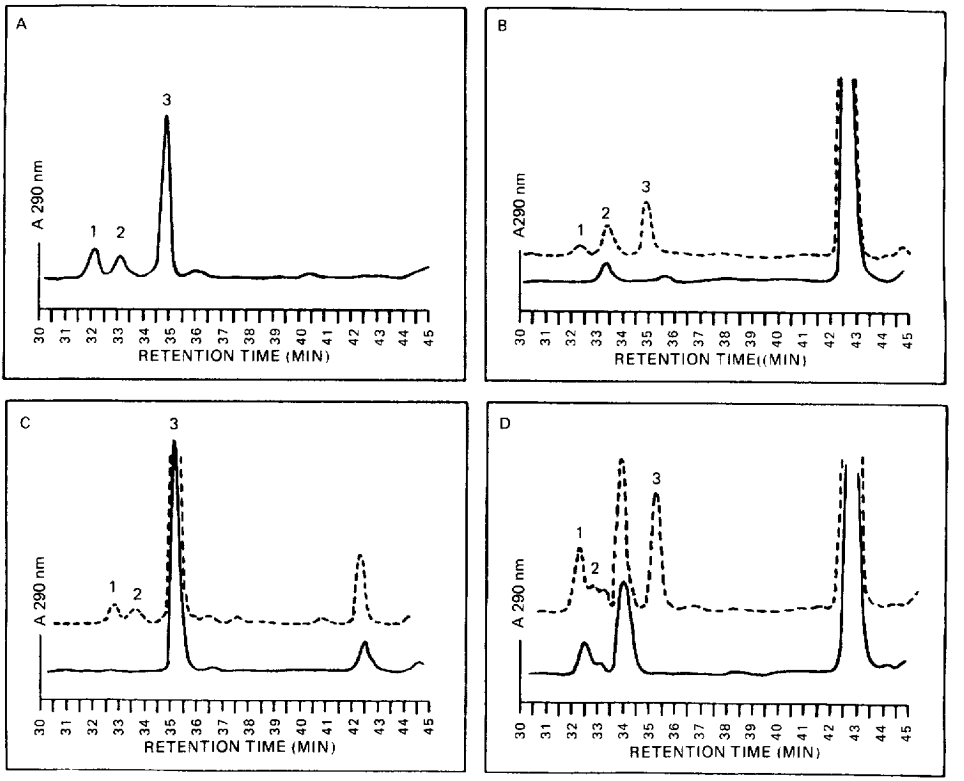

Fig. 3. Coelution of synthetic amino acid adducts and the acetylated hydrolysate of 7-bromomethylbenz[*a*]anthracene-treated histone H3 on reversed-phase HPLC. Chromatographic conditions as in Fig. 1. (A) Acetylated hydrolysate of 7-bromomethylbenz[*a*]anthracene-treated histone H3; (B) N-acetyllysine (solid line) and coinjection with H3 products (broken line); (C) N-acetylcysteine (solid line) and coinjection with H3 products (broken line), (D) N-acetylhistidine (solid line) and coinjection with H3 products (broken line). Absorbance at 290 nm was monitored; 100% absorbance is equal to 0.100 absorbance units.

der of peak 3 (Fig. 3) upon coinjection with the H3 sample (data not shown) and thus does not appear to be one of the amino acid adducts formed in the in vitro modified protein.

DISCUSSION

Our in vivo experiments with 7-bromomethylbenz[*a*]anthracene clearly indicate that all the histones are accessible for modification in mouse liver chromatin. Histone H3 is the most reactive of the inner core histones. One objective in this study was to determine how the extent of histone modification relates to the amino acid composition of the protein and the relative reactivity of specific isolated amino acids. We chose to characterize the adducts formed in vitro in order to achieve an easily detectable level of substitution (the level of substitution was less than one adduct formed per histone molecule).

There are two known H3 genes in mouse. These genes, H3-1 and H3-2, differ by a single amino acid substitution. The variant H3-1 contains two cysteines whereas H3-2 contains only one (position 96 is a serine) [8]. The amino acid composition data for H3-1 indicate that there are two cysteine, two methionine, three tyrosine, five serine, two histidine, and thirteen lysine residues among its 135 amino acids. It is interesting to note that the most reactive N-acetylated amino acid is cysteine and it appears to be the predominant adduct found in H3 in vitro (Fig. 3C). Thus, although H3 is at most 1.48% cysteine, its reactivity both in vivo and in vitro appears to be due to the highly reactive nature of the sulfhydryl, which dominates this reaction. On the other hand, lysine was only slightly reactive as an N-acetylated amino acid, yet it appears to represent a prominent site of addition in H3 in vitro (Fig. 3B). The presence of a high concentration of lysyl residues in H3 (9.73%) and the accessibility of these residues both may play a dominant role in the appearance of the lysyl adducts. The quantitation of adducts also is dependent upon the extent of hydrolysis and subsequent acetylation. We have not directly tested the effect of alkylation on these reactions, but there does not appear to be a significant decrease in adduct yield under the conditions we employ here.

In vitro we have observed an interplay between amino acid accessibility and strength of reactivity, both of which determine the sites of 7-bromomethylbenz[*a*]anthracene substitution in histone H3. The relative importance of these parameters in vivo will obviously vary for each protein, carcinogen and local environment. Even though N-acetylmethionine was the second most reactive amino acid and H3 contains two methionine residues, the methionines in H3 do not appear to react with 7-bromomethylbenz[*a*]anthracene under these conditions. In evaluating these results, it should be kept in mind that we are dealing with a single isolated histone (in the presence of dimethyl sulfoxide) and with a carcinogen that is capable of forming adducts by direct alkylation or following metabolic activation [9]. Thus the actual H3 adducts formed in vivo, where H3 is found in conjunction with the other core histones and DNA, may be quite different from those observed in these studies. The identities of the H3 adducts have been tentatively assigned based upon the coinjection of standards and spectral characteristics. To definitively assign adduct identities other techniques such as mass spectral or nuclear magnetic resonance analysis will be required.

We also describe here a reversed-phase chromatographic separation of N-acetylated amino acid methylbenz[*a*]anthracene adducts formed in vitro. This procedure may prove to be generally applicable for the separation of other polyaromatic hydrocarbon amino acid adducts. It may be noted that the acetylated adducts elute slightly ahead of the unacetylated ones (Fig. 1). It is presumed that the acetylated adducts are slightly more polar (owing to the net negative charge) and thus would be less retained than the zwitterionic form of the non-acetylated adduct.

Extension of these studies to an analysis of adduct formation in vivo may be particularly important in light of the noted changes in histone associations in transcriptionally active chromatin: Solomon et al. [10] have reported that histone H4 exhibits fewer protein–DNA contacts in heat-shocked active genes. Other studies [11,12] have indicated that the normally buried cysteine residue of histone H3 is accessible to chemical probes in transcriptionally active chromatin. Depending upon the transcriptional state of the chromatin, one might expect different extents of histone modification or different sites of amino acid substitution.

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