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Binding of Benzo[a]pyrene to Histones and Altered Affinity of Modified Histone 1 for Deoxyribonucleic Acid†

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ABSTRACT: The covalent binding of benzo[a]pyrene (B[a]P) to acid-extractable chromosomal proteins and the subsequent effect on histone 1–DNA interaction have been characterized in a model system by utilizing calf thymus nuclei as targets and rat liver microsomes as an exogenous source of enzymes for the metabolic activation of B[a]P. A two-step ion-exchange chromatography and desalting procedure was employed for removing noncovalently bound B[a]P and other contaminants. Fluorography of acetic acid–urea and Triton–acetic acid–urea–polyacrylamide gels indicated that H1 and H3 were the only principal histone targets in [^{3}H]B[a]P-modified calf thymus nuclei. The validity of this assignment was confirmed by comparison of the chromatographic distributions of [^{3}H]B[a]P cpm among peptides derived from the $HClO_4$-soluble (H1) and $HClO_4$-insoluble (core histones) protein fractions to the distributions obtained for authentic individual histone fractions. Comparison of amino acid compositions in individual peptide fractions which bound [^{3}H]B[a]P differentially yielded some insight into the probable target amino acid residues for B[a]P binding. On the basis of electrophoresis in polyacrylamide gels, it appeared as if B[a]P had bound to multiple subfractions of H1 and H3. The equivalent distribution of covalently attached [^{3}H]B[a]P among the major peptides of H1 and H3 modified either in intact nuclei or while free in solution implied that the relative accessibility of major portions of the H1 and H3 molecules for covalent B[a]P binding is not affected by interactions with DNA or other chromosomal proteins. Covalent attachment of [^{3}H]B[a]P to purified H1 reduced the affinity of this histone for DNA–cellulose.

Despite progress in understanding the metabolic activation of chemical carcinogens to highly reactive forms which bind covalently to nucleophilic atoms in cellular DNA, RNA, and protein (Miller & Miller 1974; Phillips & Sims, 1979; Weinstein et al., 1978; Allfrey & Boffa, 1979), the nature of the critical cellular target(s) is not yet known. A commonly held view is that chemically induced cancer is caused by somatic mutation, i.e., by a heritable alteration in DNA sequence. However, several observations inconsistent with this theory have led to consideration of alternative, epigenetic mechanisms of transformation: (1) Normal differentiation involves stable changes in phenotype resulting from variable gene activity, i.e., transcriptional control (Davidson, 1976). (2) The correlation between carcinogenic potential and mutagenic potential is both qualitatively and quantitatively incomplete. Not all mutagens are carcinogens and not all carcinogens are mutagens (McCann et al., 1975; Miller & Miller, 1971; Marquardt et al., 1977; Furth, 1975; Segaloff, 1975). Strong mutagens are not necessarily strong carcinogens and vice versa (Sivak, 1976; Wood et al., 1975; Levin et al., 1975). Chemically induced transformation is a more frequent event (10^2–10^3 times) than chemical mutagenesis (Parodi & Brambilla, 1977; Marquardt, 1980). (3) Some cancers undergo spontaneous reversion to normal tissues (ganglioneuroblastoma) (Dyke & Mulkey, 1967). (4) Several types of malignant cells retain the ability to undergo differentiation into a variety of apparently normal tissues (Pierce & Wallace, 1971; Ilmensee & Mintz, 1976; McKinnell et al., 1976).

Because of the possible epigenetic origin of cancer, we have studied the binding of the ubiquitous environmental carcinogen B[a]P[1] to nuclear proteins. In this paper we describe the binding of metabolically activated B[a]P to histones and demonstrate an altered interaction of modified H1 with DNA. Histones are integral structural components of chromatin and have a dynamic role in several aspects of gene function (Lilley & Pardon, 1979; Mathis et al., 1980). Binding of the bulky B[a]P group to histones could interfere with accurate reading of the DNA template during transcription or replication. The effect could be long lasting because of the low turnover of core histones and possible lack of repair of carcinogen damage to polypeptides (Allfrey & Boffa, 1979). B[a]P binding could

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[1] Abbreviations: B[a]P, benzo[a]pyrene; PMSF, phenylmethanesulfonyl fluoride; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; HMG proteins, high mobility group proteins; Gdn·HCl, guanidine hydrochloride; IAA, iodoacetamide; $NaDodSO_4$, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; CNBr, cyanogen bromide; PPO, 2,5-diphenyloxazole.

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simulate or block an essential target for normal secondary chemical modification. In view of the proposed requirement for specific combinations of such modifications during changes in gene function (Johnson & Allfrey, 1978) and the sensitivity of histone–macromolecular interactions to chemical perturbation (Spiker & Isenberg, 1977; Alder et al., 1974; Szopa et al., 1980; Fasy et al., 1979), the potentially disruptive effect is apparent. Our choice of calf thymus nuclei as a target system was dictated in part by the extensive biochemical and biophysical characterization of the histones isolated from this tissue as well as by other considerations (Jahn & Litman, 1979). The highly conserved structures, and even interaction strengths, of histones throughout evolution (Isenberg, 1979) suggest that binding to the histones of calf thymus will be analogous to histone binding in other in vivo target tissues.

Experimental Procedures

Modification of Intact Calf Thymus Nuclei with Metabolically Activated [^{3}H]B[a]P and Extraction of Chromosomal Proteins. [^{3}H]B[a]P at a specific activity of 25–66 Ci/mmol was obtained from New England Nuclear. Nuclei (non-Triton washed) were prepared as described (Jenson et al., 1980) and washed twice without PMSF–propanol. Lower specific activity [^{3}H]B[a]P nuclear modification reactions used in the gel filtration and ion-exchange procedures were carried out as described (Jahn & Litman, 1979) in a 5-mL reaction volume containing 5×10^8 nuclei, 2.5 mCi of [^{3}H]B[a]P, 1 mg/mL microsomes, and 1 mM NADPH. Following modification with [^{3}H]B[a]P, 4.5×10^9 additional unmodified nuclei were added. Nuclei and chromatin were then washed, and histones were extracted with 0.2 M H_2SO_4 as described (Jenson et al., 1980) with the addition of a 0.35 M NaCl–PMSF chromatin wash prior to the H_2SO_4 extraction. Noncovalently bound [^{3}H]B[a]P and other contaminants were then removed by ion-exchange chromatography and desalting as described below. This procedure results in the covalent attachment of approximately 1 molecule of [^{3}H]B[a]P per 10 000 molecules of histone. Higher specific activity nuclear modification reactions used for fluorography of polyacrylamide gels were the same as lower activity modifications except the amount of [^{3}H]B[a]P was increased to 10 mCi, no additional unmodified nuclei were added, and the 0.35 M NaCl wash was eliminated.

Modification of Purified Individual Histone Fractions with Metabolically Activated [^{3}H]B[a]P. The [^{3}H]B[a]P modification of purified H1 and H3 (prepared as described below) was performed in an equilibrium dialysis apparatus to minimize possible contamination of the histones with acid-soluble microsomal proteins shed during the modification reaction. The apparatus contained two 1-mL compartments (1.9 cm diameter interface × 0.4 cm deep) separated by a treated dialysis membrane (Spectrapor No. 2 in the case of H1 and Spectrapor No. 1 in the case of H3). Side A of the apparatus contained 5 mg of purified H1 or H3 in 1 mL of 0.25 M sucrose, 0.01 M Tris-HCl, 2% ethanol, and 0.2 mM NADPH, pH 7.4. Side B contained 1–5 mCi of [^{3}H]B[a]P and 0.2 mg of microsomal protein in the same solution. Following incubation at 37 °C for 1 h with agitation at 200 cycles/min, side A, containing the [^{3}H]B[a]P-modified histones, was made 0.2 M in H_2SO_4 and filtered through a 0.45 μm pore Millipore filter. Noncovalently bound [^{3}H]B[a]P and other contaminants were then removed by ion-exchange chromatography and desalting as described below. Fluorography of [^{3}H]B[a]P-modified individual histone fractions subjected to electrophoresis in polyacrylamide gels showed all detectable [^{3}H]B[a]P to be associated with the appropriate histone band.

Removal of Noncovalently Bound [^{3}H]B[a]P and Other Contaminants by Ion-Exchange Chromatography and Desalting. To 1 part of the 0.2 M H_2SO_4 extracts were added 1 part of 0.4 M NaOH, 2 parts of 0.1 M sodium phosphate, 10 M urea, pH 7.0, and concentrated DTT, making the final solution 0.05 M Na_2SO_4, 0.05 M sodium phosphate, 5 M urea, and 1 mM DTT, pH 7.0 (equilibration buffer A). This sample was applied to a column of Bio-Rex 70 in equilibration buffer A, and the column was washed until no absorbance at 260 or 230 nm and no ^{3}H cpm were detected. The histones, the HMG proteins, protein A24, and small amounts of other proteins were eluted with 5 M Gdn·HCl and 1 mM DTT. The eluted proteins were desalted on a column (15 times the sample volume) of Bio-Gel P-4 in 0.02 M HCl and lyophilized. The P-4 resin was washed with absolute ethanol to remove adsorbed [^{3}H]B[a]P after each use.

Reduction and Alkylation of Protein Sulfhydryls. Proteins were eluted from the Bio-Rex 70 "cleanup" column as described above but with 7 M Gdn·HCl, 1 M Tris-HCl, pH 8.0. The solution was then flushed with nitrogen and reduced with DTT (0.25 mg/mg of protein) for 3 h at 37 °C. IAA at 1.5-fold molar excess over DTT was added, and the solution was flushed with nitrogen and incubated at 23 °C for an additional 2 h. Desalting was carried out on Bio-Gel P-4 in 0.02 M HCl, and the proteins were lyophilized.

Isolation of Highly Purified H1 and H3. H1 and the HMG proteins were isolated from H_2SO_4 extracts of calf thymus chromatin by futher extraction with 0.75 M $HClO_4$ (Johns, 1977) and separated as described (Sanders, 1977). Small amounts of HMG's remaining in the H1 preparation was eliminated by gel filtration on a 1.5 × 420 cm column of Bio-Gel P-60 (Böhm et al., 1973). For isolation of H3, the $HClO_4$ pellet was resuspended in 1 mM HCl, dialyzed against 1 mM HCl, and lyophilized. A 500-mg portion was resuspended in 25 mL of 0.7 M Gdn·HCl, 0.1 M sodium phosphate, and 1 mM DTT, pH 7.0, and applied to a 1.6 × 27 cm column of Bio-Rex 70. The column was washed with the same solution until no absorbance at 230 nm was detected. H2A and H2B were eluted with a step of 1.0 M Gdn·HCl in the same buffer. After further washing H3 and H4 were eluted with 5 M Gdn·HCl in the same buffer. Following dialysis and lyophilization of the material eluted with 5 M Gdn·HCl, highly purified H3 was isolated as the oxidized dimer by chromatography on a 1.5 × 420 cm column of Bio-Gel P-60 (Böhm et al., 1973). Purified H1 and H3 appeared homogeneous in three polyacryalmide gel electrophoresis systems (see below), and their amino acid compositions were in close agreement with those reported elsewhere (Johns, 1977; Jenson et al., 1980).

Polyacrylamide Gel Electrophoresis and Fluorography. Proteins were electrophoresed in slab gels (1.5 mm × 28 cm) containing (1) 15% acrylamide, 2.5 M urea, and 0.95 M acetic acid (Panyim & Chalkley, 1969), (2) 12% acrylamide, 6 mM Triton X-100, 7.5 M urea, and 0.88 M acetic acid (Zweidler, 1978), or (3) 12% acrylamide, 0.1% $NaDodSO_4$, and 0.373 M Tris-HCl, pH 8.8 (LeStourgeon & Beyer, 1977). Samples for gels 1 and 2 were reduced with DTT and alkylated with IAA as described above. Samples for the $NaDodSO_4$ gels were reduced immediately prior to electrophoresis by incubating in pH 7.4 sample buffer containing 0.1 M DTT under a nitrogen atmosphere at 37 °C for 2 h. The temperature was maintained at 20 °C during electrophoresis to control temperature-dependent mobility shifts, and Coomassie blue staining, fluorography (Bonner & Laskey, 1974), and photography through a 5507-Å filter utilized standard procedures.

Chemical Cleavage of H1, Unfractionated Core Histones, and H3. The 0.75 M $HClO_4$ soluble (H1) portion of an H_2SO_4 extract of [^{3}H]B[*a*]P-modified intact nuclei was chromatographed on a 15 × 420 cm column of Bio-Gel P-60 (Böhm et al., 1973) to remove trace amounts of HMG proteins not eliminated in the earlier 0.35 M NaCl chromatin extraction step. In one set of parallel experiments, (1) the P-60 column fractions containing H1 and (2) [^{3}H]B[*a*]P modified, purified H1 were cleaved at tyrosine with NBS and the resulting cleavage products were separated on Sephadex G-100 as described (Sherod et al., 1974). In a second set of parallel experiments, (1) the $HClO_4$-insoluble (core histone) portion of an H_2SO_4 extract of [^{3}H]B[*a*]P-modified intact nuclei and (2) a mixture of [^{3}H]B[*a*]P-modified, purified H3 and unmodified core histones were reduced (Houghten & Li, 1979) to eliminate oxidized forms of methionine resistant to cleavage by CNBr. Digestion with CNBr and separation of the resulting peptides on Sephadex G-50 fine were carried out as described (DeLange et al., 1973). Column fractions were lyophilized and resuspended in 1 mL of 5 M Gdn·HCl (Pierce) in order to determine absorbance at 230 nm prior to scintillation counting. $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr peptides indicated that our procedures cleaved H3 at close to 100% efficiency. The identities of CNBr peptides of H3 resolved on G-50 fine were determined by amino acid analysis in the case of CNBr I and by amino acid sequencing in the case of CNBr II [[^{14}C](carboxymethyl)-cysteine was detected in the butyl chloride extraction of sequencer step 6] (DeLange et al., 1973). CNBr III was not identified.

DNA–Cellulose Chromatography. Double-stranded DNA–cellulose was prepared from Sigma type V calf thymus DNA and Bio-Rad Cellex 410 as described (Alberts & Herrick, 1971) with the addition of a UV irradiation step (Litman, 1968). The chromatographic matrix contained 0.42 mg of DNA/mL of packed cellulose as determined by digestion with DNAse I and hydrolysis with 0.5 M perchloric acid (Stein, 1978). For chromatography, the method of Alberts & Herrick (1971) was adapted for gradient elution of H1. Purified H1 was modified with metabolically activated [^{3}H]B[*a*]P. A 3-mg portion was dissolved in 1 mL of 0.02 M Tris-HCl, 1 mM EDTA, and 10% glycerol, pH 8.1 (equilibration buffer B), containing 0.01 M NaCl and applied to a 0.9 × 58 cm column of DNA–cellulose in the same solution. The column was eluted at 4 °C, first with a 200-mL linear gradient of 0.01–0.40 M NaCl in equilibration buffer B and then with a 200-mL linear gradient of 0.40–0.65 M NaCl in equilibration buffer B. H1 was eluted with the second gradient. The concentration of NaCl in the eluted fractions was determined by conductivity.

Additional Methods. Amino acid composition analysis and automated N-terminal sequencing were carried out as described previously (Jenson et al., 1980).

Results

Removal of Noncovalently Bound [^{3}H]B[a]P and Other Contaminants from [^{3}H]B[a]P-Modified Proteins. Figure 1 illustrates the effectiveness of the procedure used to eliminate noncovalently bound [^{3}H]B[*a*]P and additional nonhistone contaminants from 0.2 M H_2SO_4 extracts of calf thymus nuclei which were modified by metabolically activated [^{3}H]B[*a*]P. An H_2SO_4 extract was applied to a column of Bio-Rex 70 in equilibration buffer A (Figure 1a). After the column was washed with equilibration buffer A, the histones, protein AK, protein A24, residual HMG proteins, and other basic proteins along with about 4% of the DNA present in the H_2SO_4 extract

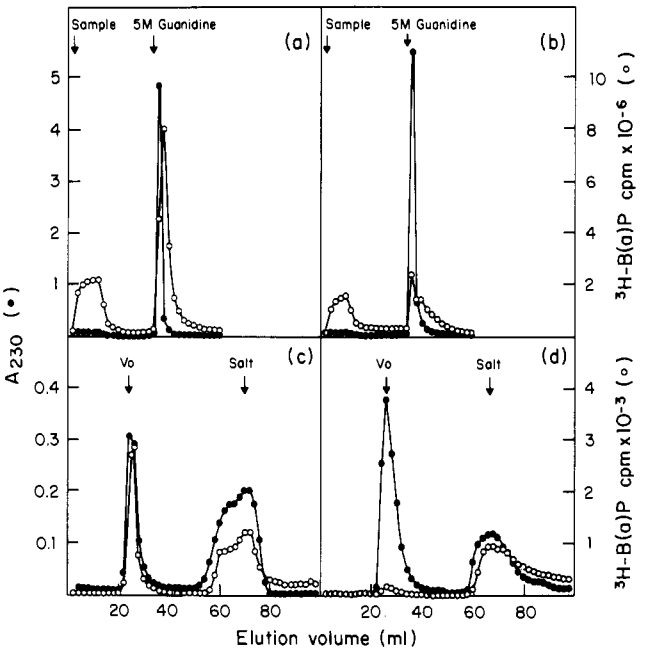

FIGURE 1: Removal of noncovalently bound [^{3}H]B[*a*]P from [^{3}H]-B[*a*]P-modified proteins. A 2.5-mg portion of a 0.2 M H_2SO_4 extract of [^{3}H]B[*a*]P-modified calf thymus nuclei containing 6.8 × 10^7 cpm was applied to a 0.7 × 3 cm column of Bio-Rex 70 in equilibration buffer A (a). After the column was washed with equilibration buffer A, the histones and lesser amounts of other proteins were eluted with 5 M Gdn·HCl. The 5 M Gdn·HCl eluate (~12 mL) was applied directly to a 2.5 × 35 cm column of Bio-Gel P-4 in 0.02 M HCl (c). [^{3}H]B[*a*]P-modified proteins were recovered from the void volume (c). In a parallel experiment, equivalent amounts of unmetabolized (noncovalently bound) [^{3}H]B[*a*]P and protein were applied to identical columns of Bio-Rex 70 (b) and Bio-Gel P-4 (d). Fraction volumes were 2 mL for (a) and (b) and 5 mL for (c) and (d).

were eluted with 5 M Gdn·HCl. The material in the column breakthrough consisted of NADPH from the modification reaction, most of the DNA present in the H_2SO_4 extract, and trace amounts of protein of net acidic amino acid composition (consisting of ~60 bands in $NaDodSO_4$–polyacrylamide gels). The protein eluted from the Bio-Rex 70 column with 5 M Gdn·HCl was applied directly to a desalting column of Bio-Gel P-4 (Figure 1c), and the [^{3}H]B[*a*]P-modified protein was recovered in the void volume at close to 100% yield. The [^{3}H]B[*a*]P adducts in the Bio-Rex 70 breakthrough and P-4 salt volume were not identified. Figure 1b,d illustrates the results of a parallel experiment in which equivalent amounts of unmetabolized (noncovalently bound) [^{3}H]B[*a*]P and histone were applied to the same columns. When employed in series, these two columns removed >99.9% of the free [^{3}H]-B[*a*]P through its interaction with the hydrophobic chromatographic matrices. Comparison of the left-hand side of Figure 1 ([^{3}H]B[*a*]P metabolically activated) with the right-hand side (ummetabolized, free [^{3}H]B[*a*]P) indicates that most of the metabolically activated [^{3}H]B[*a*]P that was eluted with protein was covalently attached (>97%). In addition, repeated precipitation with acetone (Johns, 1977) of histones from the void volume of Figure 1c failed to remove significant quantities of the protein-bound [^{3}H]B[*a*]P. In all subsequent procedures, noncovalently bound B[*a*]P was eliminated by employing this two-column procedure.

Detection of [^{3}H]B[a]P Target Proteins by Fluorography of Polyacrylamide Gels. Histones were extracted from calf thymus nuclei which had been modified with metabolically activated [^{3}H]B[*a*]P and reduced with DTT and alkylated with IAA to eliminate oxidized forms of H3 which exhibited a broad range of electrophoretic mobilities. [^{3}H]B[*a*]P-modified

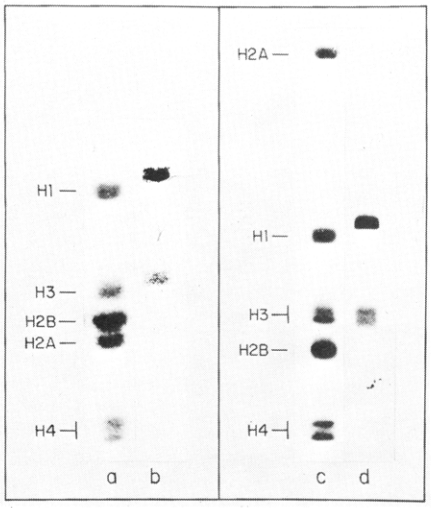

FIGURE 2: Fluorography of [^{3}H]B[*a*]P-modified histones. Histones were extracted from [^{3}H]B[*a*]P-modified calf thymus nuclei, cleared of unbound [^{3}H]B[*a*]P, and reduced and alkylated. A portion of the protein containing 10 000–15 000 cpm was electrophoresed in slab gels (1.5 mm × 28 cm) containing 15% acrylamide, 2.5 M urea, and 0.95 M acetic acid (a; b) or 12% acrylamide, 6 mM Triton X-100, 7.5 M urea, and 0.88 M acetic acid (c; d). Gels were stained with Coomassie blue, impregnated with PPO, and dried (a; c). The dried, PPO-impregnated gels were exposed to X-ray film for 4 weeks at −70 °C (b; d). The alignment of the X-rays and gels is accurate to within approximately 5% of the width of the individual histone bands.

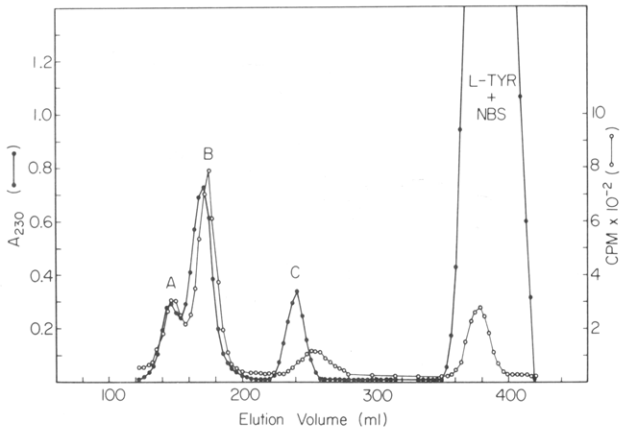

FIGURE 3: Chromatography of NBS peptides of [^{3}H]B[*a*]P-modified H1 on Sephadex G-100. Histones were extracted with H_2SO_4 from [^{3}H]B[*a*]P-modified calf thymus nuclei. H1 was obtained by chromatography of a 0.75 M $HClO_4$ extract of the [^{3}H]B[*a*]P-modified histones on Bio-Gel P-60. Additional unmodified H1 was added to give a total of 15 mg of protein and digested at its single tyrosine with NBS. The mixture of peptides was separated on a 2.4 × 145 cm column of Sephadex G-100 in 0.05 M HCl. Fraction volume was 3 mL. An indistinguishable profile was obtained with purified H1 that had been modified with [^{3}H]B[*a*]P while free in solution.

histones were electrophoresed in polyacrylamide gels containing acetic acid–urea (Figure 2a) or Triton X-100–acetic acid–urea (Figure 2c), and the gels were subjected to fluorography (Figure 2b,d) to determine the distribution of [^{3}H]B[*a*]P among the proteins. In both gel systems, bands of radioactivity electrophoresed in the vicinity of H1 and H3, although their mobilities were shifted. When equal quantities of unmetabolized (noncovalently bound) [^{3}H]B[*a*]P and histone were electrophoresed and fluorographed in an identical fashion, no bands of radioactivity were detected. Fluorography of [^{3}H]B[*a*]P-modified histones separated on $NaDodSO_4$ gels was consistent with [^{3}H]B[*a*]P binding to H1 (not illustrated). The identity of the core histone (H3, H2B, H2A, and H4) target was not clear from $NaDodSO_4$ gels because of the lower resolution of these components in this gel system.

Although the clarity of resolution is somewhat reduced in Figure 2a,c as a result of the PPO impregnation procedure, both the acetic acid–urea and Triton–acetic acid–urea gel systems resolved subfractions of H1 and H3. The fluorographs in parts b and d of Figure 2 indicate that [^{3}H]B[*a*]P had bound to at least two subfractions of H1 and H3, respectively. In the latter case, [^{3}H]B[*a*]P appears to have bound at a higher degree to an H3 band with a slower electrophoretic mobility, possibly corresponding to a more highly acetylated form (Cousens et al., 1979). While the altered mobilities of the [^{3}H]B[*a*]P-modified proteins relative to the unmodified histones could represent binding to subfractions or differentially modified forms of the histones, this seems unlikely. The occurrence of the multiple X-ray fluorograph bands noted above and the similarity in overall width of the X-ray fluorograph and stained gel bands suggest, rather, that [^{3}H]B[*a*]P had bound to most (or all) subfractions and modified forms of the histones and had caused a uniform change in their electrophoretic mobilities. Another possibility would be that the [^{3}H]B[*a*]P had actually bound to minor components that were labeled to a high specific activity and that had electrophoretic mobilities similar to, but not identical with, those of H1 and H3. Because of the latter possibility, additional analytical methods were used to confirm these results.

Gel Filtration of [^{3}*H*]*B*[*a*]*P-Modified Peptides.* In an effort to corroborate the binding of [^{3}H]B[*a*]P to H1, [^{3}H]B[*a*]P-modified histones (modified while in intact nuclei) were extracted with 0.75 M $HClO_4$, and the supernatant containing H1 was chromatographed on Bio-Gel P-60 (Böhm et al., 1973). The column fractions containing purified H1 and the associated [^{3}H]B[*a*]P peak were pooled and cleaved at tyrosine with NBS, and the resulting fragments were separated by gel filtration (Figure 3). Amino acid analysis and overall elution behavior confirmed that peaks A, B, and C contained uncleaved H1, the C-terminal peptide, and the N-terminal peptide of H1, respectively (Sherod et al., 1974). The elution characteristics of [^{3}H]B[*a*]P relative to A_{230} were consistent with the identification of H1 as a principal target molecule. An indistinguishable profile was obtained with purified H1 that had been modified with metabolically activated [^{3}H]B[*a*]P in an equilibrium dialysis cell, suggesting that, under the conditions of modification used here, the distribution of accessible target amino acid residues is the same for H1 in chromatin as for purified H1 free in solution. When equal quantities of unmetabolized (noncovalently bound) [^{3}H]B[*a*]P and NBS-digested H1 were chromatographed in an identical fashion, no peaks of radioactivity were found.

For corroboration of the binding of [^{3}H]B[*a*]P to H3, the distributions of [^{3}H]B[*a*]P in peptides derived from [^{3}H]B[*a*]P-modified, purified H3 vs. that in peptides of [^{3}H]B[*a*]P-modified, unfractionated core (non-H1-containing) histones were compared. [^{3}H]B[*a*]P-modified histones (modified while in intact nuclei) were extracted with $HClO_4$ to remove H1. The insoluble core histone pellet was cleaved with CNBr, reduced and alkylated to eliminate disulfide forms of the cysteine-containing peptides, and subjected to gel filtration (Figure 4a). Ninety-eight percent of the counts were distributed among four peaks: 63% was recovered in the void volume containing CNBr I, the major N-terminal peptide of H3; 6% was recovered in a smaller peak eluting on the trailing edge of the void volume; 9% was recovered in a peak containing CNBr II, the cysteine-containing peptide of H3; 20% was recovered in the salt volume. In a parallel experiment, purified H3 was modified with [^{3}H]B[*a*]P, digested with CNBr, reduced and alkylated, and separated on the same chromatog-

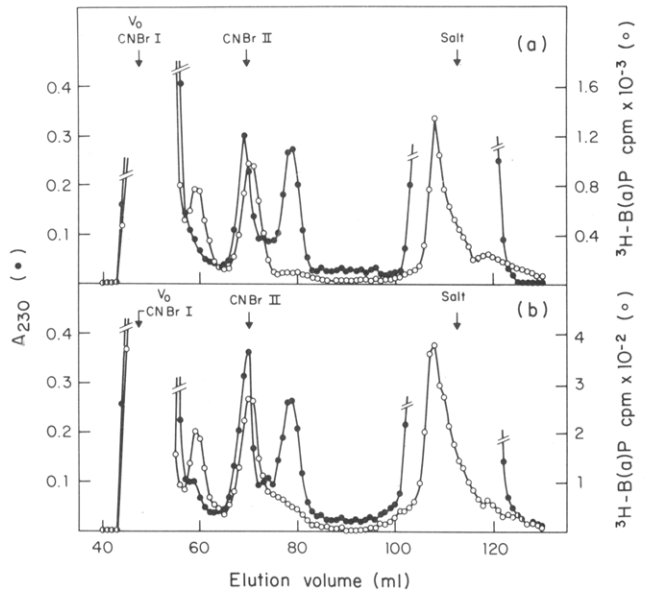

FIGURE 4: Chromatography of CNBr peptides of [^{3}H]B[*a*]P-modified core histones and [^{3}H]B[*a*]P-modified, purified H3. A 6-mg portion (200 000 cpm) of core histones, extracted from [^{3}H]B[*a*]P-modified intact nuclei, was digested with CNBr, reduced and alkylated, and applied to a 0.9 × 200 cm column of G-50 fine in 30% acetic acid (a). In a parallel experiment, purified H3 was modified in solution with [^{3}H]B[*a*]P. A 1-mg portion of the modified, pure H3 containing 60 000 cpm was mixed with 6 mg of unmodified core histones, digested with CNBr, reduced and alkylated, and applied to the same column of G-50 fine (b). Fraction volume was 1.2 mL.

raphy system (Figure 4b). Comparison of parts a and b of Figure 4 illustrates that the distribution of counts among the CNBr peptides was the same in both cases, confirming H3 as the principal core histone target.

Chromatography of [^{3}H]B[a]P-Modified H1 on DNA–Cellulose. Purified calf thymus H1 was modified with metabolically activated [^{3}H]B[*a*]P, applied to a column of DNA–cellulose, and eluted with a linear gradient of NaCl (Figure 5). H1 was eluted from the DNA column between 0.40 and 0.55 M NaCl in agreement with other reports (Fasy et al., 1979). The major peak of [^{3}H]B[*a*]P cpm was eluted at a lower concentration of NaCl (0.03 M less) than the major peak of absorbance, suggesting that [^{3}H]B[*a*]P binding had reduced the affinity of H1 for DNA–cellulose. High, decreasing levels of absorbance early in the chromatogram were the result of the elution buffer. [^{3}H]B[*a*]P-modified forms of H1 did not contribute to the absorbance profile (~1 out of 3000 molecules of H1 contained B[*a*]P). Smaller peaks of [^{3}H]B[*a*]P cpm occurring before the 100-mL elution volume chromatographed in the salt volume of Bio-Gel P-4 and, therefore, did not represent B[*a*]P bound to H1. In a parallel experiment employing plain cellulose (no DNA), the counts and absorbance peaks coeluted in the column breakthrough, indicating that the early elution of [^{3}H]B[*a*]P-modified H1 was not the result of a differential interaction with the chromatographic matrix.

Four areas of the DNA–cellulose absorbance peak were pooled and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5, inset). The $NaDodSO_4$ gel profiles indicate that chromatography on DNA–cellulose had partially separated the major H1 subtypes. This result suggested a possible alternative explanation for the observed early elution of [^{3}H]B[*a*]P, i.e., preferential binding to H1 subtype(s) with lower affinities for DNA–cellulose. So that this possibility could be tested, unfractionated [^{3}H]B[*a*]P-modified H1 was analyzed by $NaDodSO_4$ gel electrophoresis (Figure 6a) and

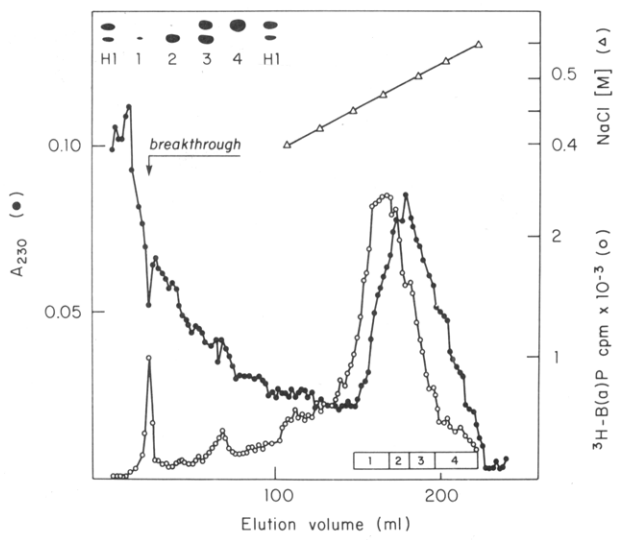

FIGURE 5: Chromatography of [^{3}H]B[*a*]P-modified H1 on DNA–cellulose. Purified H1 was modified in solution with [^{3}H]B[*a*]P. A 3-mg portion (2.3×10^6 cpm) in 1.5 mL of 0.02 M Tris-HCl, 1 mM EDTA, and 10% glycerol, pH 8.1 (equilibration buffer B), containing 0.01 M NaCl was applied to a 0.9 × 58 cm column of DNA–cellulose in the same solution. The column was eluted at 4 °C with two successive linear gradients containing 0.01–0.40 M NaCl and 0.40–0.65 M NaCl in equilibration buffer B. Fraction volume was 2 mL. The concentration of NaCl in eluted fractions was determined by conductivity (Δ). Numbered boxes at the bottom of the figure represent areas of the chromatogram that were pooled for analysis by polyacrylamide electrophoresis in slab gels (1.5 × 28 cm) containing 12% acrylamide, 0.1% $NaDodSO_4$, and 0.373 M Tris-HCl, pH 8.8 (inset). Gel wells containing unfractionated [^{3}H]B[*a*]P-modified H1 prior to chromatography are indicated as "H1" (inset).

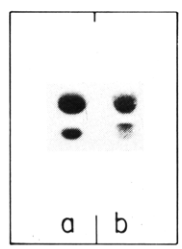

FIGURE 6: Fluorography of [^{3}H]B[*a*]P-modified H1. A 15-μg portion of the [^{3}H]B[*a*]P-modified H1 used for DNA–cellulose chromatography ("H1", Figure 5 inset) was electrophoresed in $NaDodSO_4$ slab gels (conditions as in Figure 5), stained with Coomassie blue, impregnated with PPO, and dried (a). The dried, PPO-impregnated gel was exposed to X-ray film for 4 weeks at −70 °C (b).

fluorography (Figure 6b). The fluorographic results were not consistent with such a high degree of differential B[*a*]P binding. The relative abundance of [^{3}H]B[*a*]P cpm in the trailing $NaDodSO_4$ gel H1 subtype (Figure 6b) contrasts with the very low counts/absorbance ratio of DNA–cellulose column pool 4 which contains primarily the same H1 subtype (Figure 5). Similarly, the relatively high counts/absorbance ratio in DNA–cellulose column pools 1 and 2 is not reflected in the levels of [^{3}H]B[*a*]P binding to this H1 subtype. In addition, partial separation of three [^{3}H]B[*a*]P-modified H1 subtypes by ion-exchange chromatography on Bio-Rex 70 (Bonner et al., 1968) indicated comparable levels of B[*a*]P binding to the subtypes resolved (not illustrated).

Discussion

Distribution of [^{3}H]B[a]P among the Five Histones of Calf Thymus. In order to characterize the distribution of covalently attached [^{3}H]B[*a*]P among chromosomal proteins, it was necessary to remove [^{3}H]B[*a*]P that was bound tightly through noncovalent, hydrophobic interactions. The ion-exchange/

desalting cleanup operation on Bio-Rex 70 and P-4 resulted in the removal of over 99.9% of the free [^{3}H]B[*a*]P, the major portion having been retained by the acrylamide (P-4) and methacrylic acid–divinylbenzene (Bio-Rex 70) chromatographic matrices. Possible ionic interactions between a charged metabolite of [^{3}H]B[*a*]P and protein would most likely have been disrupted by the 5–7 M Gdn·HCl used to elute the histones from Bio-Rex 70 and, if not retained by the matrices, would have eluted with the Gdn·HCl in the salt volume of the P-4 column. In addition, control experiments emphasized the ability of the analytical chromatography and electrophoresis supports to eliminate further free B[*a*]P. Since this work focused on the attachment of [^{3}H]B[*a*]P to histones, it also was desirable to eliminate nonhistone macromolecules containing potentially reactive nucleophilic atoms. Chromatography on Bio-Rex 70 was used to remove most of the DNA and many nonhistone proteins from the acid extracts of chromatin. In addition, multiple analytical procedures were employed in order to eliminate contributions by low-level contaminants modified at a high specific activity by [^{3}H]B[*a*]P. The latter approach proved important as (1) many minor proteins were detected in the acid extracts and (2) in most cases radioactivity distributions did not align precisely with optical absorption or gel staining characteristics of histones.

In acid extracts of [^{3}H]B[*a*]P-modified intact calf thymus nuclei, H1 and H3 were found to be the principal histone targets for covalent [^{3}H]B[*a*]P binding; no covalent binding to H2A, H2B, or H4 was detected by the methods employed. Fluorography employing polyacrylamide gel electrophoresis and gel filtration of NBS peptides demonstrated the occurrence of [^{3}H]B[*a*]P-modified H1. Fluorography suggested that H3 was the only additional histone target. In order to confirm that most of the [^{3}H]B[*a*]P not bound to H1 was bound to H3, the chromatographic distributions of [^{3}H]B[*a*]P among CNBr peptides derived from the $HClO_4$-insoluble histones were compared to those obtained for an authentic H3 fraction. This comparison confirmed that all detectable non-H1 [^{3}H]B[*a*]P cpm were the result of binding to H3. Additional confirmation was obtained by rechromatography of peaks of [^{3}H]B[*a*]P-modified, reduced H3 and [^{3}H]B[*a*]P-modified, oxidized H3 on Bio-Gel P-60. In this case all non-H1 peaks of cpm were generated by redistribution of the two H3 peaks throughout the chromatogram (not illustrated).

Fluorography of [^{3}H]B[*a*]P-modified histones electrophoresed in acetic acid–urea gels demonstrated that [^{3}H]B[*a*]P had bound to at least two subfractions of H1. Similarly, fluorography of Triton–acetic acid–urea gels indicated binding to at least two subfractions of H3. With the latter, [^{3}H]B[*a*]P was bound at a greater specific activity to a slower moving, possibly more highly acetylated form. Preferential binding of [^{3}H]B[*a*]P to a more highly acetylated form of H3 would be consistent with the findings that H3 acetylated in vitro has a reduced affinity for chromatin (Wong & Marushige, 1976) and that histones having lower affinities for chromatin are generally more accessible to certain (lysine) chemical probes (Wong & Marushige, 1976; Tack & Simpson, 1979).

Other laboratories have examined the binding of B[*a*]P to histones. Bresnick et al. (1977) and Pezzuto et al. (1978) reacted nuclei from rat tissues with microsomally activated [^{3}H]B[*a*]P and reported peaks of radioactivity with mobilities in polyacrylamide gel electrophoresis similar to those of H1 and H4 and additional, unresolved peaks in vicinity of H3/H2B/H2A. Kootstra & Slaga (1979) and Kootstra et al. (1979) reacted duck erythrocyte oligonucleosomes and nuclei with [^{14}C]B[*a*]P-diol epoxide (anti) and reported bands of [^{14}C]B[*a*]P with mobilities in $NaDodSO_4$ gels similar to those of H3, H2B, H4, and an unidentified component. This group also reacted hamster embryo cells in culture with the parent hydrocarbon and two metabolites of [^{3}H]B[*a*]P. MacLeod et al. (1980) reported that the relative distribution of radioactivity among gel bands with mobilities similar to those of H1, H3, and H2A depended on the form of B[*a*]P used. Whitlock (1979) reacted HeLa cell core particles with ^{14}C-labeled diol epoxide (anti) and found bands of [^{14}C]B[*a*]P in $NaDodSO_4$ gels with mobilities similar to those of all of the core histones with the binding pattern dependent upon the ionic strength during modification. Comparison of these results is complicated by differences in the source of the chromatin, the nature of the activated B[*a*]P, and the limited resolving ability and singular nature of the analytical systems employed. Several observations emphasize the need for a multifaceted analytical approach; e.g., an oxidized form of H3, protein AK (Jenson et al., 1980), and a subfraction of H1 coelectrophorese in $NaDodSO_4$ gels (unpublished) as do a form of H2A and H3 (West & Bonner, 1980). In addition, carcinogen binding may alter the mobility of a protein in a variety of electrophoretic and chromatographic systems (this work). Although H1 and H3 are the probable targets of B[*a*]P binding in the calf thymus model system, differences may occur with other tissue sources and activation procedures.

[^{3}H]B[a]P Distribution in Peptides of Histones That Had Been Modified in Intact Nuclei vs. in Solution. The radioactivity distributions obtained for peptides of H1 and H3 modified by [^{3}H]B[*a*]P in intact nuclei or in solution were indistinguishable, indicating that the relative distribution of accessible target amino acid residues is the same. The results also imply that the relative accessibility of major portions of the H1 and H3 molecules for covalent [^{3}H]B[*a*]P binding is not affected by specific interactions of these molecules with DNA and other proteins.

[^{3}H]B[a]P Binding to Specific Amino Acid Residues. The amino acid composition of H1 which includes 27% lysine but no cysteine, methionine, or histidine (Cole, 1977), other potential nucleophilic targets (Miller & Miller, 1974), suggests that the ϵ-amino group of lysine may be the principal site of binding. The distribution of [^{3}H]B[*a*]P among the NBS peptides of H1 (Figure 3) is consistent with this possibility. The C-terminal peptide contains 79% of the lysines (Cole, 1977) and 78% of the bound [^{3}H]B[*a*]P, and the N-terminal peptide contains 21% of the lysines and 22% of the [^{3}H]B[*a*]P. Direct confirmation of lysine as the predominant target amino acid residue in H1 has been obtained by chemical modification of H1 prior to [^{3}H]B[*a*]P binding (unpublished). Comparison of the [^{3}H]B[*a*]P vs. amino acid distributions in the CNBr fragments of H3 does not permit this type of conclusion. It was possible, however, to eliminate cysteine as a principal target residue since the major N-terminal peptide of calf thymus H3, CNBr I, which contains no cysteine, bound $\sim$7 times as much [^{3}H]B[*a*] as CNBr II, which contains both of the cysteines.

Effect of [^{3}H]B[a]P Binding on the Affinity of H1 for DNA–Cellulose. [^{3}H]B[*a*]P-modified H1 was eluted from DNA–cellulose at a lower concentration of NaCl than unmodified H1. Comparable levels of [^{3}H]B[*a*]P binding to multiple subtypes of H1 indicate that this was the result of a weakened affinity of H1 for DNA and not preferential binding to early eluting subtypes. Although this phenomenon occurred in a narrow concentration range, the difference in elution molarity between [^{3}H]B[*a*]P-modified and unmodified

H1 (30 mM NaCl) was 3 times the difference observed for in vitro phosphorylated calf thymus H1 (10 mM NaCl) which exhibits a looser binding to DNA–Sephadex than native H1 (Fasy et al., 1979). In vivo phosphorylation of H1 (Fisher & Laemmli, 1980) also causes a reduction in binding to both single-stranded and native DNA–cellulose. These phosphorylations modulate the influence of H1 on chromatin structure and function (Johnson & Allfrey, 1978). Our results indicate that B[*a*]P modification can have an effect similar to this naturally occurring secondary chemical modification of H1, i.e., alteration of its affinity for DNA. Although the biological significance of this effect remains to be demonstrated, these findings form a basis for potential epigenetic perturbation of cellular function which may occur during the course of polycyclic aromatic hydrocarbon induced carcinogenesis.

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