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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CHROMATIN HISTONES

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SUMMARY

A method is described for the rapid analysis of histones by high-performance liquid chromatography on reversed-phase μBondapak columns, containing either octadecylsilane (C_{18}) or cyanopropylsilane (CN) bonded to silica particles packed in either steel columns or Radial-Pak cartridges. A linear gradient progressing from water–acetonitrile (80:20) to water–acetonitrile (40:60) and increasing in acetonitrile concentration at the rate of 10%/h was used to elute the histones at a flow-rate of 1 ml/min for steel columns or 2 ml/min for Radial-Pak cartridges. Two conditions were found to be necessary to achieve histone fractionation: (1) silylation of the active silanol groups on the silica matrix, and (2) 0.1–0.3% trifluoroacetic acid in the eluting solvent. More than 95% of the total [^{3}H]lysine-labeled protein applied to the CN column was eluted. The histone fractions were identified by their electrophoretic mobilities in both acid–urea and Triton DF-16 polyacrylamide gels. Histones were eluted from the columns in the following order: H1, H2B, (LHP)H2A, (MHP)H2A, H4, (LHP)H3, and (MHP)H3 (where LHP and MHP refer to the less-hydrophobic and more-hydrophobic histone variants). Phosphorylated and acetylated histone molecules were not separated from their unmodified parent molecules. The volatile nature of the water–acetonitrile–trifluoroacetic acid eluting solvent facilitated recovery of salt-free histones from the fractions by direct lyophilization of the column effluents. The best resolution of histone fractions was obtained with the Radial-Pak μBondapak C_{18} cartridge using 0.3% TFA. However, for analytical studies, the best detection was obtained by using the μBondapak CN steel column. Poorer resolution was obtained by using the non-silica based PRP-1 reversed-phase column, containing a polystyrene-divinylbenzene resin under the same conditions.

INTRODUCTION

The chromatin of cells consists of DNA, histone proteins, non-histone proteins, and RNA. The histones are known to function as structural components of the chromatin, organizing the DNA into nucleosomes[1]. The histones may also serve a variety of regulatory functions for such DNA activities as gene replication during S phase, chromosome formation, chromosome separation during mitosis, and gene

transcription[2–13]. These regulatory functions are thought to occur as a result of substitution of one histone variant for another, or by modifying the histones through processes involving phosphorylation and acetylation.

To study these histone functions, it is necessary to isolate the histones from the chromatin and then to fractionate the histones into their five classes (H1, H2A, H2B, H3, and H4). The most detailed studies will also require further fractionation of these classes into the histone variants which have slightly different primary structures and into the different phosphorylated and acetylated post-synthetic modified forms. These fractionations are usually accomplished by conventional electrophoresis[14–16] or column chromatography[3,17–19].

With the advent of high-performance liquid chromatography (HPLC), it was hoped that these laborious and time-consuming conventional methods could be replaced by a simple, rapid, high-resolution HPLC method. However, many problems were encountered which discouraged the application of HPLC technology to histones. These problems centered primarily around the irreversible adsorption of the histones on HPLC column packings. In this report we describe a set of column packings and elution solvents which have overcome these problems and are suitable for fractionating histones by HPLC with high recovery. An abstract of this work has been previously reported[20].

EXPERIMENTAL

Cell cultures and histone preparation

Chinese hamster cells (line CHO) were grown exponentially in suspension culture as described previously by Tobey *et al.*[21]. Chromatin was prepared from either whole-cell homogenates or isolated nuclei as described by Gurley *et al.*[22]. Histones were extracted from the chromatin with 0.4 *N* sulfuric acid, recovered by acetone precipitation, and stored as a lyophilized powder at −20°C until used for HPLC[22].

To label histones with radioisotopes, CHO cells were grown exponentially for 2–3 generations in 1-l cultures containing either 50 μCi of [^{3}H]lysine or 50 μCi of [^{14}C]lysine as described previously[22,23]. The ^{3}H-acetylation of histones in the [^{14}C]lysine-labeled cells was then accomplished by exposing the cells to 20 mCi of [^{3}H]acetate for 20 min[23]. The ^{32}P-phosphorylation of histones in the [^{3}H]lysine-labeled cells was accomplished by exposing the cells to 20 mCi of $H_3{}^{32}PO_4$ for 1 h[22,23]. Following the radioactivity labeling period, histones were prepared as described above.

High-performance liquid chromatography

Reversed-phase HPLC was performed on histones using several different columns manufactured by Waters Assoc. (Milford, MA, U.S.A.): (1) 8C1805 Radial-Pak® C_{18} cartridge (10 cm × 8 mm I.D.) containing 5-μm spherical porous (125 Å) packing of octadecylsilane bonded to μPorasil® (an irregular 125-Å porous silica, the μBondapak® C_{18} steel column (30 cm × 3.9 mm I.D.) containing 10-μm particle packing of octadecylsilane bonded to μPorasil® (an irregular 125-Å porous silica) the silanol groups of which had been end-capped[24,25] by silylation with trimethylchlorosilane[26] by the manufacturer; (3) μBondapak CN steel column (30 cm × 3.9 mm I.D.) containing cyanopropylsilane bonded to end-capped silica packing; (4)

μBondapak Phenyl steel column (30 cm × 3.9 mm I.D.) containing phenylethylsilane bonded to end-capped silica packing; (5) μBondapak NH_2 steel column (30 cm × 3.9 mm I.D.) containing aminopropylsilane bonded to end-capped silica packing; (6) 8MBC1810 Radial-Pak μBondapak C_{18} cartridge (10 cm × 8 mm I.D.) containing 10-μm particle packing of octadecylsilane bonded to end-capped silica packing; and (7) Radial-Pak μBondapak CN cartridge (10 cm × 8 mm I.D.) containing 10-μm particle packing of cyanopropylsilane bonded to end-capped silica packing. Reversed-phase HPLC was also performed on histones by using a Hamilton (Reno, NV, U.S.A.) PRP-1® steel column (15 cm × 4.1 mm I.D.) packed with a 10-μm particle size, rigid, spherical, macroreticular resin of polystyrene–divinylbenzene.

Lyophilized histones were prepared for HPLC by dissolving them in water containing 0.05–0.3% trifluoroacetic acid (TFA; Sequanol grade; Pierce, Rockford, IL, U.S.A.). After equilibration of the column with the initial chromatography solvent, 140 μl of histone solution containing 400–500 μg of protein were applied to the column. The histones were then chromatographed at a flow-rate of 1 ml/min through the steel columns, or 2 ml/min through the Radial-Pak cartridges, using a linear gradient of acetonitrile (UV grade, Burdick & Jackson Labs., Muskegon, MI, U.S.A.) progressing from water–TFA to acetonitrile–TFA (the TFA concentration in the water and acetonitrile was the same as the TFA concentration in the histone sample and was constant throughout the solvent gradient). The initial and final percentage of acetonitrile in the elution gradients varied with experimental design and is reported for each experiment.

The sample and gradient were delivered to the column by either a Perkin-Elmer Series 3 liquid chromatograph or a Waters Model 6000A solvent delivery system equipped with a Model 720 system controller. Two pumps were used in both systems, one to deliver the water–TFA and the other to deliver the acetonitrile–TFA. Histones eluted from the column were detected by UV absorption at 206 nm by either a Perkin-Elmer LC-55 variable-wavelength spectrophotometer (a photomultiplier instrument) or a Waters Lambda-Max Model 480 spectrophotometer (a photodiode instrument).

Radioassay and electrophoretic analysis of histones fractionated by HPLC

The HPLC column effluent was collected in 1-ml fractions for either radioassay or electrophoresis. The radioactivity of these fractions was counted in a Packard Tri-Carb Model 3320 liquid scintillation spectrometer as described previously[22,23]. The samples to be used for electrophoretic analysis were frozen, lyophilized to dryness, and stored at −20°C.

Electrophoresis of each HPLC fraction was performed on 25 × 0.5 cm polyacrylamide gels by two different methods: (1) the Panyim and Chalkley acid–urea gel system, which separates the five classes of histones[27], and (2) the Zweidler Triton DF-16® gel system, which separates histone variants according to their hydrophobic properties[28]. The specific conditions used in our laboratory for the acid–urea system and the Triton DF-16 system have been described by Gurley *et al.*[15] and Halleck and Gurley[11], respectively. Following electrophoresis the gels were stained with Amido Black 10B, destained by diffusion, and photographed[22,23].

RESULTS AND DISCUSSION

Adsorption of histones to HPLC column packings

In our laboratory, we attempted to fractionate histones on a variety of HPLC columns[22]: Hydrogel IV®, Corasil II®, controlled-pore glass (CPG-10®), Silica A/10®, DEAE-cellulose, Bondapak C_{18}/Porasil-B, and Bondapak C_{18}/Corasil. In this work, several problems were encountered: (1) irreversible adsorption of histones on column packings; (2) poor detection of histones by UV absorption at 240–280 nm; (3) inability to resolve the histone classes adequately; and (4) inability of column packings to withstand the pressures involved in HPLC. Other laboratories had experienced problems also. Using LiChrosorb Si 60® (a 10-μm silica gel column) and a methanol–acetic acid–water elution solvent, Pickart and Thaler[29] obtained several unidentified fractions from commercially prepared histones. However, the similar retention times of these fractions in lysine-rich and arginine-rich histone preparations indicated that resolution of the histone classes was insufficient for most applications. Certa and Von Ehrenstein[30] succeeded in obtaining ten fractions from calf thymus histones by using a Hypersil® C_{18} column with an elution gradient progressing from 100% 0.1 *M* $NaClO_4$, 0.1% H_3PO_4, pH 2.1, to 60% acetonitrile. In that case, however, low recovery of histones from the column limited the application of the procedure, especially in metabolic experiments requiring quantification.

As a result of all these experiences, it was obvious that efforts to develop an HPLC method for histone fractionation should concentrate on achieving (1) high recovery of histones from the column packings, and (2) resolution of all five classes of the histones. Reports by Bennett *et al.*[31], Hermodson and Mahoney[32], and Henderson *et al.*[33], that peptides and proteins could be eluted from reversed-phase columns in high yield by using gradients of organic solvents containing TFA, led us to investigate the fractionation of histones in such a system[20,22,23].

Since histones contain very few UV-absorbing amino acid residues, detection of histones in column effluents by UV absorption at 240–280 nm is not sufficiently sensitive. For this reason it was necessary to monitor the column effluents at 206 nm where the protein carbonyl groups could be detected. Initial attempts to elute histones from a Radial-Pak C_{18} reversed-phase column with a gradient of acetonitrile containing 0.3% TFA failed (Fig. 1a). Consultation with the manufacturer revealed that the silica in this reversed-phase C_{18} column had not been end-capped, and thus contained active silanol groups. When histones were loaded on a μBondapak C_{18} steel column packed with silanols that were end-capped, histones could not be eluted with an acetonitrile gradient containing no TFA (Fig. 1b), but when 0.3% TFA was present in the gradient six protein fractions were detected in the eluate from the column (Fig. 1c).

From these results, it was concluded that two different conditions must be met before histones can be eluted from reversed-phase C_{18} column packings: (1) the free silanol groups on the silica packing must be end-capped, and (2) a hydrophobic ion-pairing agent such as TFA must be present in the eluent.

Histone fractionation on μBondapak C_{18} columns

To identify the histones eluted from the μBondapak C_{18} column, 1-ml fractions of the effluent were collected, lyophilized, and subjected to electrophoresis[22].

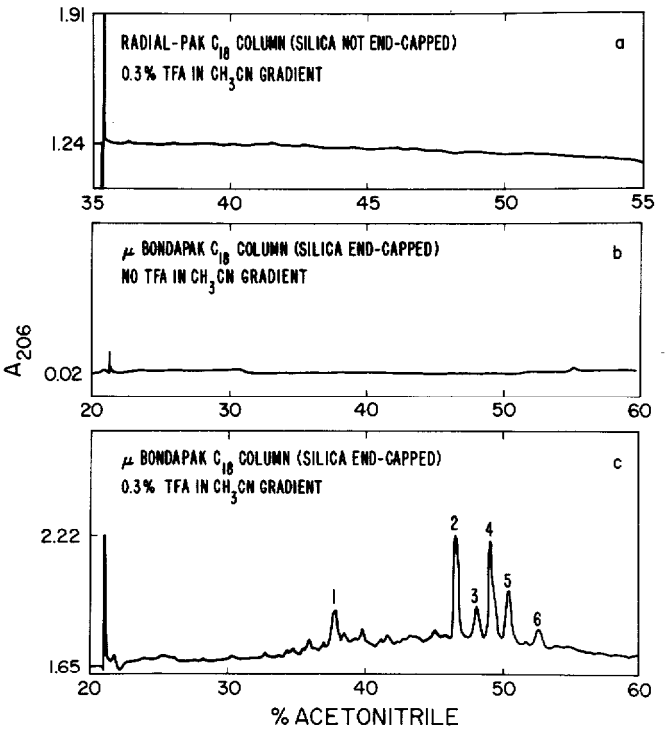

Fig. 1. Effects of end-capping and TFA on histone fractionation using silica-based C_{18} columns and acetonitrile-containing eluents. Histones (417 μg) isolated from whole-cell homogenates were loaded on the columns. (a) HPLC of histones using a Radial-Pak C_{18} column packed with silica that had not been end-capped and an elution gradient containing 0.3% TFA. The acetonitrile concentration increased at the rate of 10%/h with a flow-rate of 2 ml/min. (b) HPLC of histones using a μBondapak C_{18} steel column packed with silica that had been end-capped and an elution gradient containing no TFA. The acetonitrile concentration increased at 20%/h with a flow-rate of 1 ml/min. (c) HPLC of histones using a μBondapak C_{18} steel column packed with silica that had been end-capped and an elution gradient containing 0.3% TFA. The other gradient conditions were the same as in (b) above.

The six peaks in Fig. 2a were identified by acid–urea gel electrophoresis (Fig. 2b and 2c) and found to contain all the major histone fractions. Peaks 1 and 2 contained the lysine-rich histones H1 and H2B, respectively (Fig. 2b). Peak 3 contained H2A; peak 4 contained a mixture of H2A, H4 and a minor unidentified protein (UP) thought to be an H2A-related protein[16,22]; peaks 5 and 6 both contained H3 (Fig. 2c). The presence of H2A in peaks 3 and 4 and the presence of H3 in peaks 5 and 6 suggested that two variants of both H2A and H3 had been separated.

Electrophoresis of peaks 3–6 on Triton DF-16 gels (Fig. 2d) confirmed that peaks 3 and 4 contained the less hydrophobic (LHP) variant of H2A and the more hydrophobic (MHP) variant of H2A, respectively, while peaks 5 and 6 contained the LHP variant of H3 and the MHP variant of H3, respectively. Thus, the histones were eluted from the μBondapak C_{18} column in the following order: H1, H2B, (LHP)H2A, (MHP)H2A + UP + H4, (LHP)H3, and (MHP)H3.

H3 is the only histone which contains cysteine residues[34–36]. During the prepa-

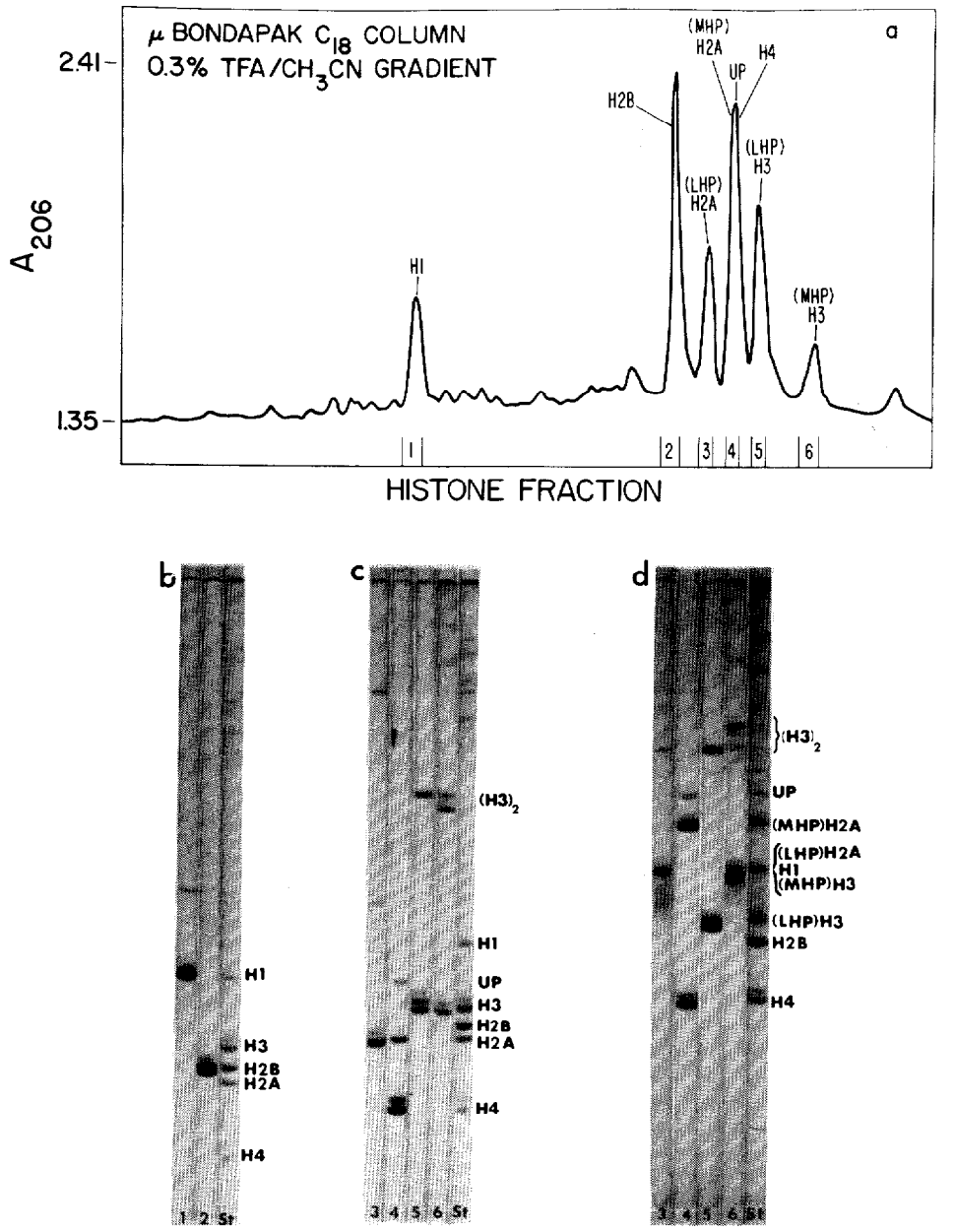

Fig. 2. Identification of histone species in the HPLC fractions from a μBondapak C_{18} steel column eluted with an acetonitrile gradient containing 0.3% TFA. (a) 3000 μg of histones isolated from CHO nuclei was subjected to HPLC as in Fig. 1c. The column effluent was collected in 1-ml fractions. The fractions indicated on the abscissa were subjected to electrophoresis. (b) Acid–urea polyacrylamide gel electrophoresis of peaks 1 and 2. (c) Acid–urea polyacrylamide gel electrophoresis of peaks 3, 4, 5 and 6. (d) Triton DF-16 polyacrylamide gel electrophoresis of peaks 3, 4, 5 and 6. The whole histone sample used for HPLC was also used as a mobility standard (St) for each electrophoresis experiment.

ration of histones, H3 molecules will form dimers and oligomers as a result of disulfide bridge formation. The (LHP)H3 from peak 5 was observed to form only dimers (Fig. 2c and 2d), indicating that this H3 variant contained only one cysteine residue, while the (MHP)H3 from peak 6 formed a dimer of different electrophoretic mobility plus oligomers (Fig. 2c and 2d), indicating that this H3 contained at least two cysteine residues. Thus, it is concluded that this HPLC method provides a rapid and preparative method for obtaining two H3 variants with different primary structures.

In order to determine the recovery of histones from the μBondapak C_{18} column, a [^{3}H]lysine-labeled histone sample containing a precisely determined amount of ^{3}H was subjected to HPLC. It was calculated from the radioactivity of the effluent that 91% of the labeled protein was recovered[22]. Since there was no significant loss of histones in this procedure, it was concluded that our first criteria for histone fractionation by HPLC had been met (*i.e.*, that the recovery must be high so that irreversible adsorption of histones on the column packing does not interfere with the interpretation of results from experiments requiring quantitative data).

Since elution of histones from the μBondapak C_{18} column required the presence of TFA in the eluting solvent, the TFA concentration dependency was investigated (Fig. 3a). It was found that similar resolution was obtained at 0.1%, 0.2%, or 0.3% TFA, although the peaks appeared to be sharper at 0.3% TFA. Curiously, while TFA is required to reverse histone adsorption on the C_{18} column (Fig. 1), increasing concentration of TFA increases the retention time of the histones on the column once the "adsorption threshold" has been overcome (Fig. 3a).

An acetonitrile gradient increasing at the rate of 20%/h was used to elute histones from the μBondapak C_{18} column in Figs. 1–3. Since peak 4 was found to contain both (MHP)H2A and H4, a gradient of 10%/h was used to determine if a more shallow gradient would resolve these two histones (Fig. 4a). This gradient tended to separate these histones, but did not resolve them sufficiently for most applications. Doubling the column length had no effect on resolving these two histones. Thus, it appeared that resolution of the (MHP)H2A and H4 histones would require either modifications of the column packing, or changes in the eluting solvent or solvent additive.

When histones prepared from the chromatin of blended whole cells were subjected to HPLC on a C_{18} column, considerable amounts of protein were eluted between H1 and H2B (Fig. 1c). Electrophoresis of these proteins indicated that they were not histones[22,23]. When histones prepared from isolated nuclei were fractionated on C_{18}, much less protein was detected in this region (Fig. 4a). Thus, the μBondapak C_{18} column will chromatograph contaminating non-histone proteins isolated during histone extraction. These observations suggest that a system for the fractionation of non-histone chromatin proteins may also be developed from this HPLC procedure.

Histone fractionation on μBondapak CN columns

Having found an HPLC system which produces a high recovery of histone fractions, we next examined a number of column packings using the same solvent system in an attempt to find a column which would resolve the (MHP)H2A from H4. Histones have two domains, a highly polar domain which is associated with DNA and a more hydrophobic domain which is associated with other histones in the nu-

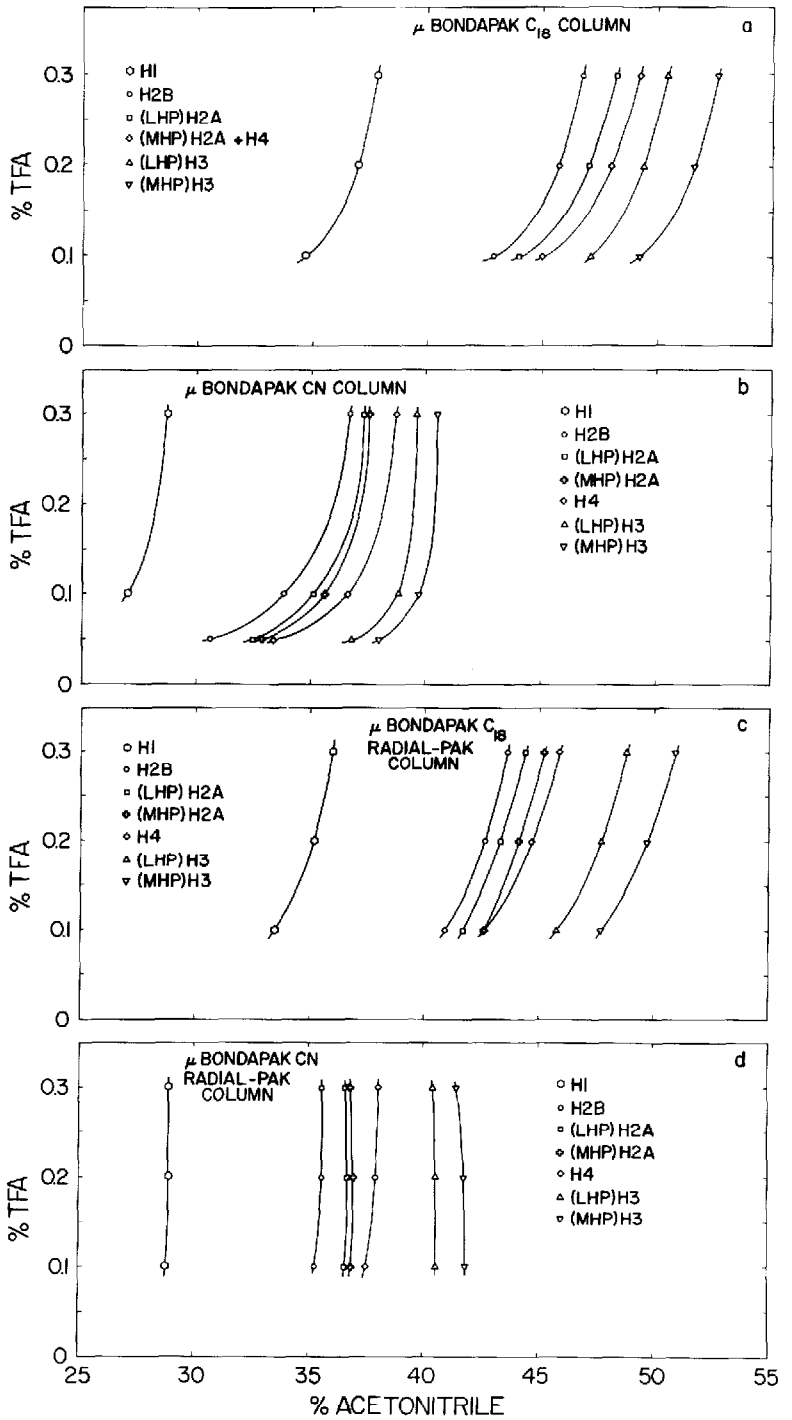

Fig. 3. Effect of TFA concentration on the retention of various histone fractions on four different μBondapak columns. (a) μBondapak C_{18} steel column eluted as in Fig. 1c. (b) μBondapak CN steel column eluted as in Fig. 4b. (c) Radial-Pak μBondapak C_{18} cartridge eluted as in Fig. 10a. (d) Radial-Pak μBondapak CN cartridge eluted as in Fig. 10b.

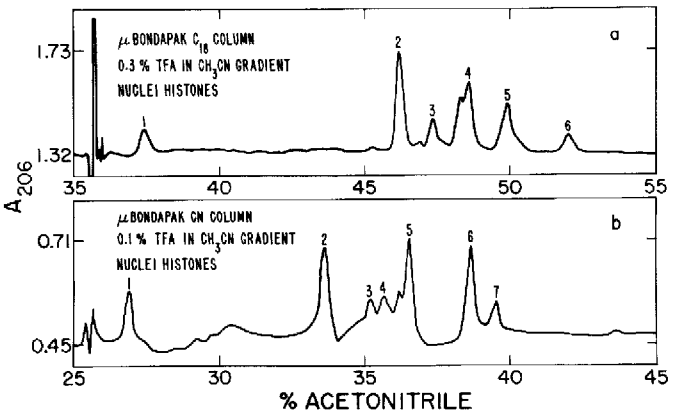

Fig. 4. Comparison of the elution of histones from two different types of μBondapak steel columns. Histones were isolated from CHO nuclei. Both columns were loaded with 417 μg of histone and eluted in the presence of TFA with an acetonitrile gradient rising at a rate of 10 %/h with a flow-rate of 1 ml/min. (a) μBondapak C_{18} column eluted with a 35–55 % acetonitrile gradient containing 0.3 % TFA. (b) μBondapak CN column eluted with a 25–45 % acetonitrile gradient containing 0.1 % TFA.

cleosome[37–39]. It is reasonable to expect that the association of histones with reversed-phase μBondapak C_{18} columns occurs through interactions of the hydrophobic parts of the molecules with the column material. It was thought that perhaps by substituting the more polar cyanopropylsilane for the highly hydrophobic octadecylsilane on the μBondapak column, the polar properties of the histones might also contribute to the histone-column interaction and thus, produce better resolution of the histone fractions[23].

To examine this possibility, histones were subjected to HPLC on a μBondapak CN column by using an acetonitrile gradient containing 0.1 % TFA (Fig. 4b). Seven peaks were resolved suggesting that perhaps (MHP)H2A had been separated from H4. In general, the histones were eluted from the CN column at considerably lower acetonitrile concentrations than they were from the C_{18} column indicating much weaker hydrophobic interactions between the histones and the CN column.

To identify the histones eluted from the μBondapak CN column, 1-ml fractions of the effluent were collected, lyophilized, and subjected to electrophoresis[23]. The seven peaks in Fig. 5a were identified as histones by electrophoresis on acid–urea gels (Fig. 5b and 5c) and on Triton DF-16® gels (Fig. 5d). (MHP)H2A was found to be separated from the H4 histone. The minor unidentified protein (UP) also separated from the H4 and was located in peak 4 with the (MHP)H2A. Thus, all the H2A class histones were separated from the H4 class, and the (LHP)H2A was partially separated from the (MHP)H2A. The histones were eluted from the μBondapak CN column in the following order: H1, H2B, (LHP)H2A, (MHP)H2A + UP, H4 (LHP)H3, and (MHP)H3.

The recovery of histones from the μBondapak CN column was determined by subjecting a [^{3}H]lysine-labeled histone sample to HPLC as described above for the C_{18} column. It was calculated that 96 % of the [^{3}H]lysine-labeled protein was recovered using 0.1 % TFA[23]. Thus, there does not appear to be any significant loss of histones on the CN column.

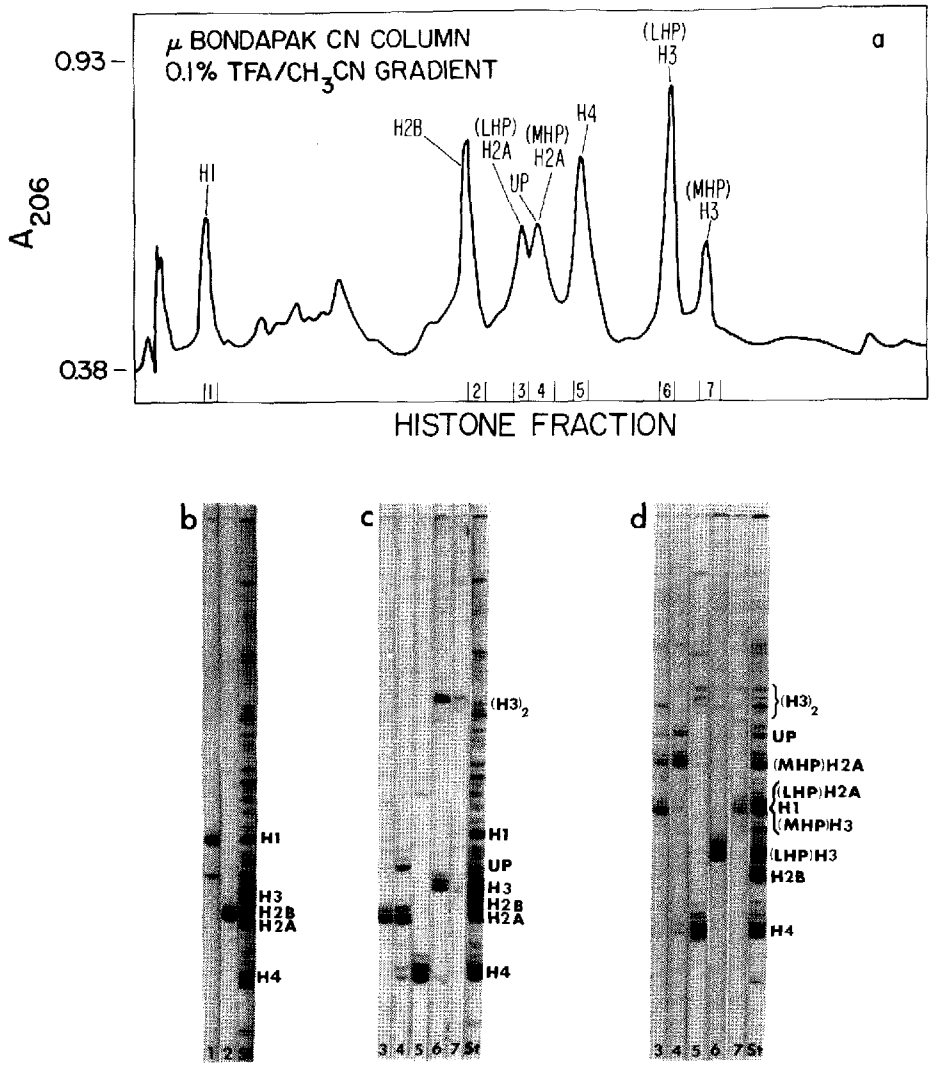

Fig. 5. Identification of histone species in the HPLC fractions from a μBondapak CN steel column eluted with an acetonitrile gradient containing 0.1% TFA. (a) 467 μg of CHO histone, isolated from whole cell homogenates was subjected to HPLC as in Fig. 4b. The column effluent was collected in 1-ml fractions. The fractions indicated on the abscissa were subjected to electrophoresis. (b) Acid–urea polyacrylamide gel electrophoresis of peaks 1 and 2. (c) Acid–urea polyacrylamide gel electrophoresis of peaks 3, 4, 5, 6 and 7. (d) Triton DF-16 polyacrylamide gel electrophoresis of peaks 3, 4, 5, 6 and 7. The whole histone sample used for HPLC was also used as a mobility standard (St) for each electrophoresis experiment.

The response of the CN column chromatography to TFA concentration (Fig. 3b) was different from the response of the C_{18} column chromatography (Fig. 3a). When 0.05% TFA was used with the CN column, H2A was eluted near the H4 (Fig. 3b) causing an overlap of (MHP)H2A with H4, similar to that observed for the C_{18} column. When the TFA concentration was raised to 0.1% TFA the two H2A variants moved to an intermediate position between H2B and H4. At 0.3% TFA,

H2A variants eluted near the H2B, resulting in an overlap of (LHP)H2A with H2B. Thus, the interaction of the H2A histones with the CN column appears to be more TFA concentration-dependent than that of H2B and H4. This dependency can be used to advantage to produce the optimum resolution of the H2A histone class on the μBondapak CN column with an acetonitrile gradient containing 0.1 % TFA.

Having found an HPLC system which produced both a high recovery of histones and resolution of all five classes of histones, we examined the μBondapak CN system to determine whether or not there was any resolution of the acetylated and phosphorylated histones from their unmodified parent molecules. Histones labeled with both [^{3}H]lysine and [^{32}P]phosphate were subjected to HPLC on the CN column. The radioactivity of 1-ml fractions of the column effluent was counted (Fig. 6). It was found that the peaks of incorporated $^{32}PO_4$ were associated with the peaks of the [^{3}H]lysine-labeled histones. Thus, there does not appear to be any resolution of the phosphorylated species from their unphosphorylated parents. Likewise, when histones labeled with [^{14}C]lysine and [^{3}H]acetate were subjected to HPLC on the CN column, the peaks of incorporated [^{3}H]acetate were associated with the peaks of [^{14}C]lysine-labeled histones (Fig. 7). Thus, there does not appear to be any resolution of the acetylated species from their non-acetylated parent compounds. From these observations it is concluded that reversed-phase HPLC of histones by the CN column system is suitable for resolving histones having primary structural differences (including class variants), but it is ineffective in resolving the various post-synthetic modifications of these histones.

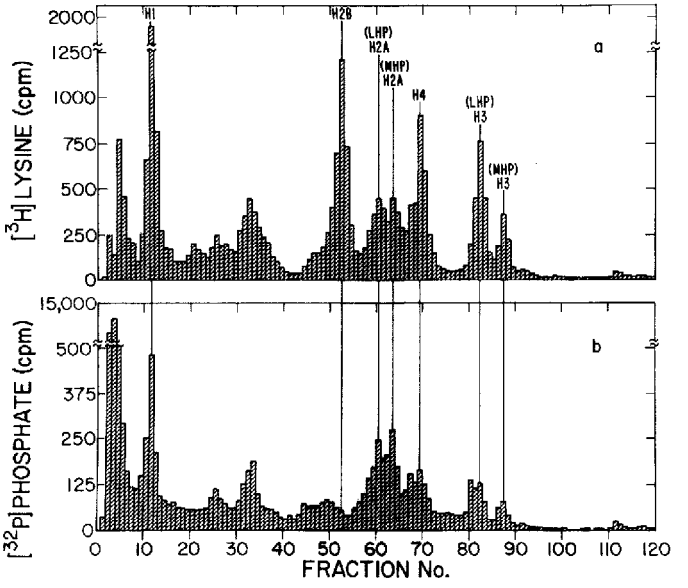

Fig. 6. Identification of phosphorylated histones eluted from a μBondapak CN steel column with 0.1 % TFA in the acetonitrile gradient. A 467-μg histone sample isolated from the whole-cell homogenate of CHO cells labeled with [^{3}H]lysine and [^{32}P]phosphate was subjected to HPLC as described in Figs. 4b and 5a. The HPLC effluent was collected in 1-ml fractions and its radioactivity was counted. (a) [^{3}H]Lysine incorporated into the primary structure of the protein. (b) [^{32}P]Phosphate incorporated into the proteins as a post-synthetic modification. Identification of the histone peaks was made from the UV absorption profile as in Fig. 5a.

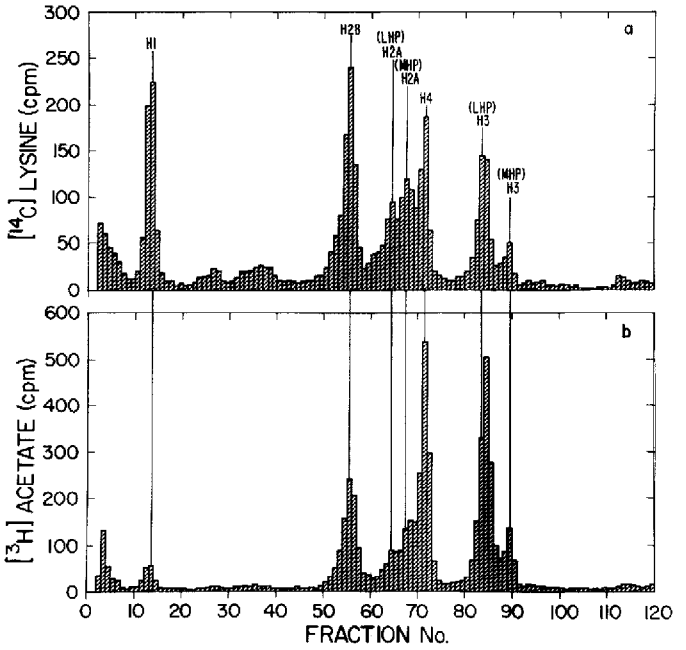

Fig. 7. Identification of acetylated histones eluted from a μBondapak CN steel column with 0.1% TFA in the acetonitrile gradient. A 467-μg histone sample isolated from the nuclei of CHO cells labeled with [^{14}C]lysine and [^{3}H]acetate was subjected to HPLC as described in Figs. 4b and 5a. The HPLC effluent was collected in 1-ml fractions and its radioactivity was counted. (a) [^{14}C]Lysine incorporated into the primary structure of the proteins. (b) [^{3}H]Acetate incorporated into the proteins as a post-synthetic modification. Identification of the histone peaks was made from the UV absorption profile as in Fig. 5a.

HPLC of histones on a μBondapak Phenyl column

Another column which sometimes provides greater resolving power for polar samples than the C_{18} column is the phenyl column[40]. To determine whether this column would have any advantages in separating (MHP)H2A from H4, or (LHP)H2A from (MHP)H2A, histones were subjected to HPLC on a μBondapak Phenyl column. To determine the elution range, a 0–100% acetonitrile gradient containing 0.1% TFA was passed through the histone-loaded column in 30 min. Large amounts of 206-nm UV-absorbing material were eluted from the column (Fig. 8a). When no histone was loaded onto the column, this material was still eluted and could be detected at either 206 nm (Fig. 8b) or at 260 nm (Fig. 8c). Thus, it appears that the phenyl column is unstable under the conditions used for histone HPLC on the C_{18} and CN columns.

HPLC of histones on a μBondapak NH_2 column

Since a better resolution of H4, (MHP)H2A, and (LHP)H2A was obtained on the more polar CN column than on the non-polar C_{18} column, it was decided to determine whether a column with even greater polarity might have an advantage over the CN column. The μBondapak NH_2 column was expected to have such properties. It has been used to chromatograph strongly polar compounds and aromatic amines[40] which have both hydrophobic and hydrophilic domains as do the histones.

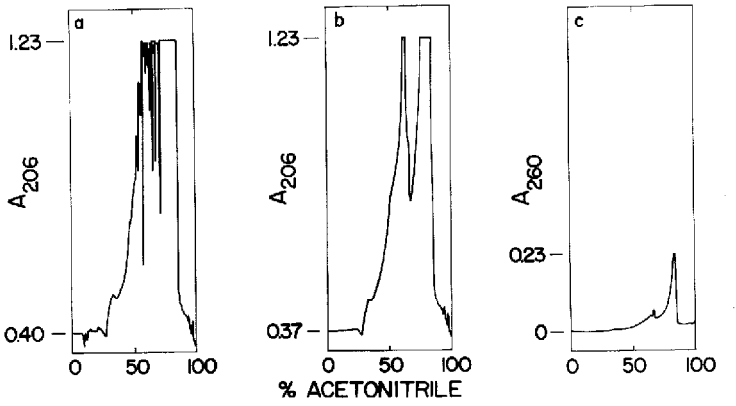

Fig. 8. Elution of a μBondapak Phenyl steel column with acetonitrile gradients containing 0.1 % TFA. (a) Absorbancy profile at 206 nm of a 467-μg histone sample eluted with a 0–100 % acetonitrile gradient in 30 min. (b) Absorbancy profile at 206 nm of a column containing no histones being eluted with a 0–100 % acetonitrile gradient in 30 min. (c) Absorbancy profile at 260 nm of a column containing no histones being eluted with a 0–100 % acetonitrile gradient in 30 min. The flow-rate was 1 ml/min in each experiment.

When histones were subjected to HPLC on the μBondapak NH_2 column using a 15–50 % acetonitrile gradient containing 0.1 % TFA, most of the protein was eluted in the void column (Fig. 9a). When a 0–20 % acetonitrile gradient containing 0.1 % TFA was used, most of the protein was still eluted in the void column (Fig. 9b).

Since TFA prevents irreversible binding of histones to the less polar C_{18} and CN columns, it was thought that perhaps TFA was not needed to perform this function in the more polar NH_2 column. (Omission of TFA from the eluting solvent would also have the advantage of decreasing the UV absorption background of the

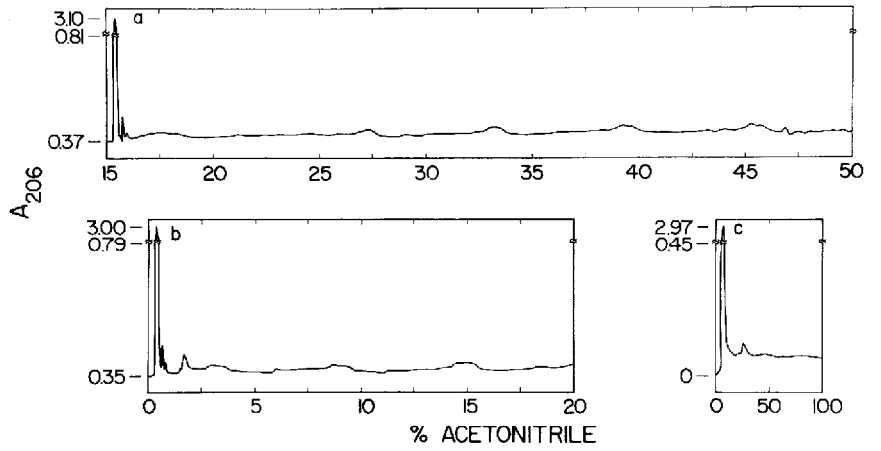

Fig. 9. Elution of histones from a μBondapak NH_2 steel column with acetonitrile gradients. The 206-nm absorbancy profile of the histones was measured while eluting the column with: (a) 15–50 % acetonitrile gradient containing 0.1 % TFA over a 210-min period; (b) 0–20 % acetonitrile gradient containing 0.1 % TFA over a 120-min period; (c) 0–100 % acetonitrile gradient containing no TFA over a 30-min period. All columns were loaded with 467 μg of histones and the flow-rate was 1 ml/min in each experiment.

eluent.) However, omission of TFA from the solvent did not prevent the elution of histones in the void volumn (Fig. 9c). Thus, it appears that, at the low pH conditions of this solvent system (pH 2), the NH_2 column is positively charged and, thus, prevents the histone interactions necessary for chromatography.

HPLC of histones on a Radial-Pak μBondapak C_{18} cartridge

Improvements in HPLC performance can frequently be obtained when a flexible walled column (cartridge) containing the stationary phase is used instead of a steel column packed with the same material. When these cartridges are placed in a high-pressure unit designed to compress the column walls radially, a uniform, dense, stable column free of void pockets and wall effects is produced[41]. This design also permits the uniform distribution of the sample across the entire top surface of the column, resulting in the narrowest possible initial bandwidth. To determine whether histone resolution could be improved in such a system, histones were subjected to HPLC on a Radial-Pak μBondapak C_{18} cartridge using 0.3% TFA in the acetonitrile elution gradient (Fig. 10a).

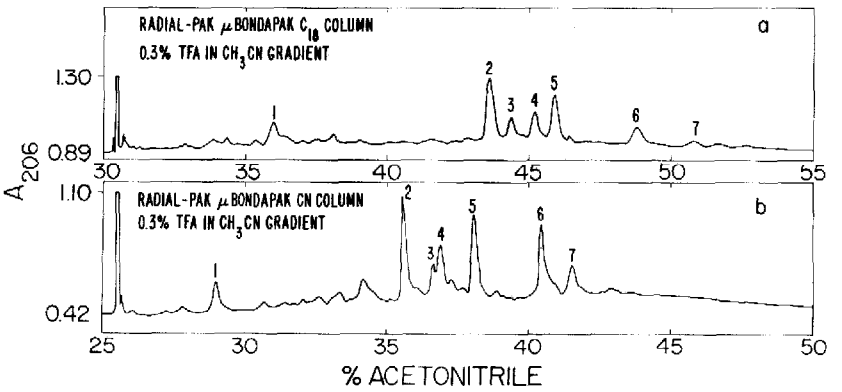

Fig. 10. Comparison of the elution of histones from two different types of Radial-Pak μBondapak cartridges. Histones were isolated from whole-cell homogenates. Both columns were loaded with 933 μg of histone and eluted with TFA containing acetonitrile concentrations that increased at a rate of 10%/h; flow-rate 2 ml/min. (a) Radial-Pak μBondapak C_{18} cartridge eluted with a 30–55% acetonitrile gradient containing 0.3% TFA. (b) Radial-Pak μBondapak CN cartridge (custom packed) eluted with a 25–50% acetonitrile gradient containing 0.3% TFA.

Significant improvement of histone resolution was obtained. While (MHP)H2A could not be separated from H4 on the steel μBondapak C_{18} column (Figs. 1c, 2a, and 4a), these two histones were separated on the Radial-Pak cartridge containing μBondapak C_{18} packing (peaks 4 and 5, Fig. 10a). Also, complete resolution of the (MHP)H2A and (LHP)H2A variants was obtained with the Radial-Pak cartridge (peaks 3 and 4, Fig. 10a) while only partial resolution was obtained for these two variants with the steel μBondapak CN column (peaks 3 and 4 of Fig. 3b; Fig. 4b).

The TFA concentration dependency for histone resolution on the Radial-Pak μBondapak C_{18} cartridge (Fig. 3c) was different from that observed with the steel μBondapak C_{18} column (Fig. 3a). Using 0.1% TFA, the (MHP)H2A and H4 histones were not resolved on either the Radial-Pak cartridge or the steel column. However, at 0.2% and 0.3% TFA these two histones were resolved on the Radial-Pak cartridge,

but not on the steel column. The separation between (LHP)H2A and (MHP)H2A was about the same on both cartridge and steel column. There was an increased retention time for both H3 variants relative to H4 on the Radial-Pak as compared to the steel column. All histones were eluted from the Radial-Pak cartridge at lower acetonitrile concentrations than from the steel column.

The reason for these differences is unclear. They may be due to (1) differences in the operating conditions of the two systems, (2) differences in column parameters (*e.g.*, surface area:void volume ratio), (3) process differences during manufacturing, or (4) a combination of these. Whatever the reason, the complete resolution of (LHP)H2A, (MHP)H2A, and H4 on the Radial-Pak μBondapak C_{18} cartridge gives this column a distinct advantage.

A curious similarity exists between the μBondapak CN steel column (Fig. 3b) and the Radial-Pak μBondapak C_{18} cartridge (Fig. 3c). In both systems, increasing the TFA concentration moves the two H2A variants from H4 toward H2B, but does not change the resolution of H2A variants significantly. Thus, the advantage of the Radial-Pak μBondapak C_{18} cartridge over the μBondapak CN steel column is based initially on better separation of (LHP)H2A from (MHP)H2A, and then on the TFA-dependent shift of these two H2A variants away from H4 as the TFA concentration is increased.

HPLC of histones on a Radial-Pak μBondapak CN cartridge

Since HPLC of histones on μBondapak CN was superior to HPLC on μBondapak C_{18}, and since HPLC on the Radial-Pak μBondapak C_{18} was significantly better than on the μBondapak C_{18} steel column, it was logical to expect that HPLC on a Radial-Pak μBondapak CN cartridge might give the best resolution. A custom-packed Radial-Pak μBondapak CN cartridge was obtained from the manufacturer, and histones were subjected to HPLC. Greater separation was obtained between the (MHP)H2A and H4 on this Radial-Pak cartridge (peaks 4 and 5, Fig. 10b) than on the steel column (peaks 4 and 5 of Fig. 4b; Fig. 5a), but no improvement was observed in the resolution of the two H2A variants (peaks 3 and 4, Figs. 4b and 10b). Thus, the Radial-Pak μBlndapak C_{18} column gave the best resolution for a complete mixture of histones.

The TFA-dependent shift of the H2A variants occured in both the steel and Radial-Pak systems using μBondapak CN (Fig. 3b and 3d). In fact, the shift of (MHP)H2A away from H4 was greater for the Radial-Pak μBondapak CN cartridge (Fig. 3d) than it was for the Radial-Pak μBondapak C_{18} cartridge (Fig. 3c). As a result, at 0.3% TFA two minor unidentified peaks were observed between (MHP)H2A and H4 (*i.e.*, between peaks 4 and 5, Fig. (10b) which could not be observed in the Radial-Pak μBondapak C_{18} system (Fig. 10a). Thus, this CN cartridge system may have a useful application in the investigation of these two proteins. It will be interesting to determine if one of them might be the presumptive minor H2A variant (UP), detected in this region of the HPLC profile by gel electrophoresis (Figs. 2 and 5), or the phosphorylated protein eluted ahead of H4 in the μBondapak CN steel column system (Fig. 6b).

HPLC of histones on a polystyrene–divinylbenzene PRP-1 column

In Fig. 1, we demonstrated that the HPLC of histones could not occur on reversed-phase columns until two conditions were fulfilled: (1) the end-capping of the

free silanols on the silica, and (2) the addition of TFA to the eluent. However, the nature of the interaction between the histones, the TFA, and the end-capped silica matrix of the packing remained unclear. For example, the TFA might interact with the histones, either as a neutral species or as an ion-pairing agent[42]; or it might not interact with the histones at all, but rather, it might solubilize histones by acting as a chaotropic agent, modifying the entropy of the solvent system[43,44]. The TFA might also interact with the silica matrix, forming a modified stationary liquid phase on the packing which was responsible for the separation of the histones. While detail studies on the nature of the TFA interactions are beyond the scope of this work, it was thought that we might be able to determine whether TFA–silica matrix interactions or histone–silica matrix interactions played any significant role in the chromatographic system after the silanols were end-capped. We could do this by simply substituting a non-silica based hydrophobic packing for the silica-based hydrophobic packing in the column.

This was accomplished by loading histones onto a steel Hamilton PRP-1 column, containing a reversed-phase spherical resin of polystyrene–divinylbenzene. This column contains no silica matrix that could possibly interact with either the TFA or the histones. It was found that histones were eluted from the column with an

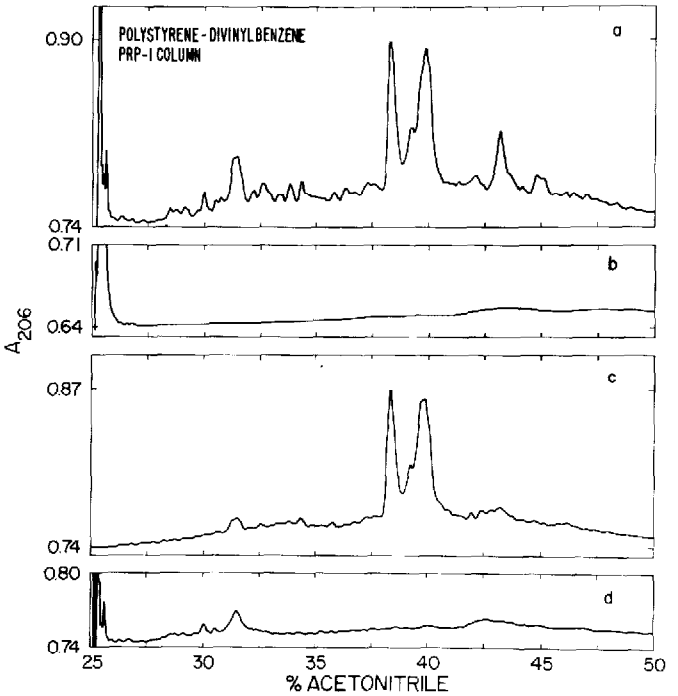

Fig. 11. HPLC of histones on a Hamilton PRP-1 steel column packed with a polystyrene–divinylbenzene spherical resin instead of a silica-based solid support. (a) 467 μg of histone isolated from whole-cell homogenates was loaded on the column and eluted with a 25–50% acetonitrile gradient containing 0.1% TFA, which was increasing in acetonitrile at a rate of 10%/h; flow-rate, 1 ml/min. (b) 467 μg of histone was loaded on the column and elution was performed with the same acetonitrile gradient but containing no TFA. (c) Without loading any additional histone on the column, following the elution in (b) above, the column was eluted with 0.1% TFA in the acetonitrile gradient. (d) The proteins in the void volume peak in (b) above were recovered by lyophilization and subjected to HPLC with 0.1% TFA in the acetonitrile gradient.

acetonitrile gradient containing 0.1 % TFA (Fig. 11a). Furthermore, the elution profile indicated that the histones were eluted in the same order as on the silica-based μBondapak columns. For example, the elution profile was similar to that observed with a μBondapak C_{18} steel column (Fig. 1c), except that (LHP)H2A was not very well separated from the (MHP)H2A + H4 peak (Fig. 11a).

When histones were loaded on the column and then subjected to HPLC with an acetonitrile gradient containing no TFA, no histones were eluted (Fig. 11b). However, when gradient elution was repeated on the same histone-loaded column with 0.1 % TFA, the histones were eluted (Fig. 11c). Because there appeared to be some loss of H1 and H3 (Fig. 11c), the material eluted in the void volume (Fig. 11b) was lyophilized to dryness and rechromatographed on the column with 0.1 % TFA. Some histone H1 and H3 were detected in this sample (Fig. 11d), indicating that some of the H1 and H3 loss shown in Fig. 11c resulted from non-absorption of these histones on the column in Fig. 11b when TFA was not used.

From these observations it is concluded that: (1) silica is not necessary for the strong adsorption of histones on reversed-phase columns: (2) the elution of histones from reversed-phase columns in general requires TFA; (3) the elution of histones in the presence of TFA is not dependent on interaction of TFA with silica; and (4) the elution order of histones from reversed-phase columns is not dependent on interactions of histones with silica. The histones are irreversibly bound to uncapped silanols on silica-based reversed-phase columns, vitiating the interactions between the histones, TFA, stationary liquid phase, and mobile liquid phase necessary for chromatography.

CONCLUSIONS

The strong adsorption of histones to HPLC column packings has prevented the application of HPLC technology to histone fractionation for several years. This adsorption results from two different sources: (1) polar interactions between the histones and the silanol groups of silica-based packings; and (2) strong hydrophobic interactions between the histones and the organic phase of the column packings. Both of these interactions must be overcome before histones can be successfully fractionated by HPLC.

The polar interactions can be overcome by either end-capping the free silanol groups on a silica-based reversed-phase column, or by using a reversed-phase column not containing silica. Once this is accomplished, the silica can no longer irreversibly adsorb the histones or influence the order of elution of histones. However, histones will still be strongly adsorbed on the organic stationary phase of the column by hydrophobic interactions. Chromatography can occur only if these hydrophobic interactions are also weakened. It was found that this can be accomplished by using TFA in the eluents.

Using a variety of μBondapak reversed-phase columns with TFA in the acetonitrile elution gradient, the histone classes were eluted in the order of their arginine content: H1, H2B, H2A, H4, and H3. Within both the H2A and H3 classes two structural variants were eluted in the order of their hydrophobicity: (LHP)H2A was eluted before (MHP)H2A, and (LHP)H3 was eluted before (MHP)H3. However, the resolution of these histones depended on both the type of stationary phase and the TFA concentration.

The best resolution was obtained with a Radial-Pak μBondapak C_{18} cartridge when 0.3% TFA was used in the acetonitrile gradient. This system should be best for preparative work, since it produces the best separation of (LHP)H2A, (MHP)H2A, and H4 and the proteins can be recovered directly by lyophilization of the volatile TFA–water–acetonitrile solvent. However, there is one problem with this system, which the user should be aware of. The high background absorption at 206 nm caused by the 0.3% TFA may cause a decrease in the sensitivity of some types of UV detectors. We found that the photodiode detectors were more affected by this problem than the photomultiplier detectors. Because of this problem, some may find that using 0.1% TFA in the water–acetonitrile solvent with a μBondapak CN column is a better system for their application, even though the resolution of (LHP)H2A and (MHP)H2A is not as good. For example, for metabolic studies where only small samples are available and quantification is required, an increased sensitivity in detection at 206 nm (resulting from the lower background of 0.1% TFA) may be necessary.

Because of this interference of detection by TFA, efforts should not be directed to finding a reagent similar to TFA which weakens the hydrophobic interactions between the histones and the organic stationary phase, yet has a low UV absorption at 206 nm.

While this system of chromatography (based on hydrophobic interactions) was found to be advantageous for fractionating histones classes and their structural variants, it was insensitive to differences in histone phosphorylation and acetylation. Since there is much concern about the biological functions of these modifications, efforts should now be directed toward finding HPLC conditions which will result in fractionation of these post-synthetic histone modifications.

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