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PREPARATION OF HISTONE VARIANTS AND HIGH-MOBILITY GROUP PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Methods have been developed for the preparation of histone variants and high-mobility group (HMG) proteins by high-performance liquid chromatography (HPLC). The individual HPLC fractions were recovered as a dry powder in 95% yield by direct lyophilization from the column effluent. Perchloric acid–soluble Hl variants and HMG proteins from Chinese hamster cells (line CHO) were separated on a μBondapak CN column using a 0–50% linear acetonitrile gradient in water containing 0.2% trifluoroacetic acid (TFA). The proteins were eluted in the following order: HMG-E/G (an HMG-14/17 class proteins from CHO cells), Hl^0, Hl, HMG-2, and HMG-1. HMG-E/G, Hl, and an unidentified protein were recovered electrophoretically pure. Hl^0 contained contaminants which could be removed by subsequent chromatography on a μBondapak C_{18} Radial-Pak® column, but HMG-1 and HMG-2 could not be completely resolved.

Nucleosomal core histones were fractionated on a μBondapak C_{18} Radial-Pak column using a 30–55% linear acetonitrile gradient containing 0.2–0.3% TFA. They were eluted in the following order: H2B, (LHP)H2A, (MHP)H2A, H4, LHP(H3), and (MHP)H3, (where LHP and MHP refer to less-hydrophobic and more-hydrophobic variants). If the gradient containing 0.3% TFA was interrupted with an isocratic elution at 43% acetonitrile, the H2B, (LHP)H2A, (MHP)H2A, and H4 proteins were completely resolved, thus providing a good preparative method for these proteins.

The H2A class of *Drosophila* histones was also fractionated on a μBondapak C_{18} Radial-Pak column using a 30–35% linear acetonitrile gradient containing 0.2% TFA. *Drosophila melanogaster* H2A, obtained as a single fraction by chromato-

graphy on Biol–Gel P-100, was eluted from the C_{18} column as three proteins. The order of elution was identified by electrophoresis to be: $H2A_{ox}$ (an oxidized form of H2A), D2 (a *Drosophila*-specific subtype), and H2A.

INTRODUCTION

It is now well established that the nucleosome is the repeating unit of chromatin structure[1]. This unit structure consists of a protein core containing two molecules each of four histones (H2A, H2B, H3 and H4), a 150–200 base-pair length of DNA, and histone H1. The DNA is wrapped around the octamer core two times and is then extended to the next core particle[2]. Histone H1 is associated with the histone core particle and with the DNA at the DNA's entry and exit sites on the core particle[2–4]. Early experiments suggested that H1 is also associated with the internucleosomal DNA linker region, but the nature of this association is not yet clear[2].

This general model is complicated by the existence of structural variants of the histones and by the possibility that structural variants of both histone H1 (such as $H1^0$) and the high mobility group (HMG) nonhistone proteins can substitute for H1 at the DNA entry and exit sites on the nucleosome[4]. Interest in the role of these variants in chromatin structure has generated a need for preparative methods for obtaining sufficient quantities of pure histone variants and HMG proteins for biochemical and structural studies.

Methods have recently been developed in our laboratory for the fractionation of histones by reversed-phase high-performance liquid chromatography (HPLC)[5–7]. These procedures are particularly useful as preparative methods, because the volatile nature of the HPLC solvents makes it possible to recover salt-free proteins from the HPLC effluents by simple lyophilization. In this report, we (a) describe the expansion of these HPLC procedures to the fractionation of HMG proteins and histone variants, and (b) determine the suitability of these preparations for biochemical and structural studies.

EXPERIMENTAL

Preparation of whole histones from Chinese hamster cells (line CHO)

Chinese hamster cells (line CHO) were grown exponentially in suspension culture, as described previously by Tobey *et al.*[8]. Chromatin was prepared from whole-cell homogenates, as described by Gurley *et al.*[5]. Unfractionated whole 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[5].

Preparation of H1, $H1^0$ and HMG proteins

Chromatin was prepared from whole-cell homogenates of CHO cells, as described previously[5]. The H1, $H1^0$, and HMG proteins were then extracted from this chromatin with 5% perchloric acid containing 0.4 *M* sodium bisulfite. These proteins were recovered by precipitation from the perchloric acid extract with 20% trichloroacetic acid[9]. The proteins were then washed with acidified acetone, dissolved in water, lyophilized to dryness, and stored at −20°C until used for HPLC. In a few

cases, these proteins were extracted from the chromatin homogenates of nuclei prepared from CHO cells in the presence of chloromercuriphenyl sulfonate[5]. These nuclei were prepared using either the nonionic detergent Nonidet P-40 (Shell Chemical Co.)[10], or a combination of Nonidet P-40 and the ionic detergent, sodium deoxycholate[10].

Preparation of H2A/D2 histones from Drosophila melanogaster

Drosophila melanogaster embryo histones were prepared as described previously by Palmer *et al.*[11]. The H2A fraction was separated from the other histones by Bio-Gel P-100 column chromatography[11]. This fraction, which contained both H2A and the Drosophila-specific D2 histone, was lyophilized for storage until used for HPLC.

HPLC

Reversed-phase HPLC of histones was performed using several different columns manufactured by Waters Assoc. (Milford, MA, U.S.A.): (1) μBondapak CN column (30 cm × 3.9 mm I.D. steel column containing cyanopropylsilane bonded to an end-capped silica packing); (2) μBondapak phenyl column (30 cm × 3.9 mm I.D. steel column containing phenylethylsilane bonded to an end-capped silica packing); and (3) μBondapak C_{18} Radial-Pak column (10 cm × 8 mm I.D. plastic cartridge containing octadecylsilane bonded to an end-capped 10-μm silica packing). During chromatography, the μBondapak C_{18} Radial-Pak column was contained in a Waters Z-Module designed to radially compress this flexible-walled plastic cartridge to produce minimum void volume and maximum efficiency.

Lyophilized histones were prepared for HPLC by dissolving them in water containing either 0.2% or 0.3% trifluoroacetic acid (TFA) (Sequanol grade, Pierce, Rockford, IL, U.S.A.). After equilibration of the column with the initial chromatography solvents, 100–200 μl of histone solution, containing 400–800 μg of protein, were applied to the column. The histones were then chromatographed at a flow-rate of 1 ml/min for the steel columns or 2 ml/min for the Radial-Pak columns, unless indicated otherwise, using a linear gradient of acetonitrile (HPLC grade; J. T. Baker, Phillipsburg, NJ, U.S.A.) progressing from water TFA to acetonitrile–TFA. The TFA concentration in the water and acetontrile was the same as the TFA concentration in the protein sample and was therefore 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 a Waters Model 6000A solvent delivery system equipped with a Model U6K injector and a Model 720 gradient system controller. Two pumps were used, one to deliver the water–TFA solvent and the other to deliver the acetonitrile–TFA solvent. The concentration of acetonitrile in the acetonitrile–TFA reservoir was 50% if the concentration in the gradient was expected not to exceed that concentration. If the gradient concentration was to exceed 50%, 100% acetonitrile, containing TFA, was used instead. The use of 50% acetonitrile is preferred, because fewer solvent mixing anomalies are observed at low acetonitrile concentrations in the solvent gradient when this prediluted solvent is used.

Proteins eluted from the column were detected by UV absorption at 206 nm using a Waters Lambda-Max Model 480 spectrophotometer. The HPLC column

effluent was collected in 1-ml fractions. The effluent from each UV absorption peak was pooled, freeze-dried, and stored at −20°C.

Electrophoretic analysis of proteins fractionated by HPLC

Electrophoresis of HPLC fractions from CHO cells was performed using two different methods: (1) the acid–urea gel system of Panyim and Chalkley[12], which separates the five classes of histones, and (2) the Tris–glycine–SDS gel system of Laemmli[13], which separates proteins according to molecular weight. One-dimensional acid–urea electrophoresis was performed in cylindrical gels and one-dimensional Tris–glycine–SDS electrophoresis was performed in slab gels, as previously described[14]. Two-dimensional gels were also employed, the first-dimension separation being made in acid–urea cylindrical gels and the second-dimension separation being made in a slab Tris–glycine–SDS gel, as previously described[15].

Electrophoresis of HPLC fractions from Drosophila histones was performed using Tris–glycine–SDS (pH 8.8) slab gels, which separates the H2A from the D2 proteins[11]. Triton DF-16 acid–urea gel electrophoresis was also used which, in addition to resolving H2A and D2, also resolves $H2A_{ox}$, the oxidized form of H2A[11].

RESULTS AND DISCUSSION

Solvent mixing

Histones elute from μBondapak reversed-phase columns in the range of 25–55% acetonitrile if TFA is present in the water–acetonitrile gradient[7]. Thus, the procedures we have previously reported for histone fractionations do not involve mixing solvents to produce low concentrations of acetonitrile[5–7]. Preliminary studies had indicated that some of the HMG proteins were eluted from these columns at lower percentages of acetonitrile[16]. Therefore, we attempted to generate a 0–55% acetonitrile gradient by mixing water 0.2% TFA with acetonitrile–0.2% TFA in order to fractionate these HMG proteins. These attempts were frustrated by the occurrence of an oscillating 206 nm-absorbing baseline which was produced below 20% acetonitrile. This oscillation had sufficient amplitude to obscure any small protein peaks being eluted in that range (Fig. 1). It was found that if a water–acetonitrile (50:50, v/v) solution containing 0.2% TFA was used to mix with water–0.2% TFA to produce the acetonitrile gradient, this oscillation was eliminated and a smooth

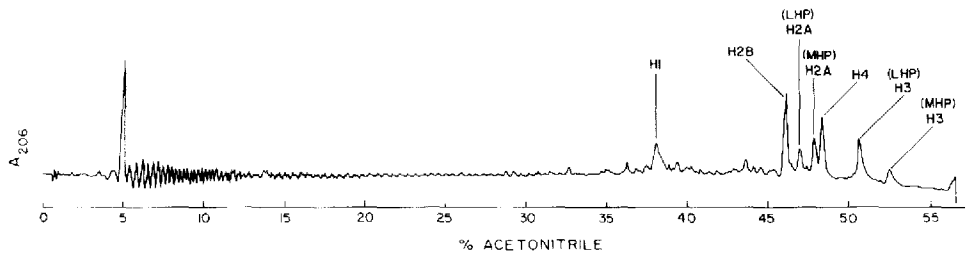

Fig. 1. Oscillating 206-nm absorbancy at low acetonitrile concentrations resulting when water–TFA is mixed with acetonitrile–TFA to form the acetonitrile gradient. CHO histones (467 μg) were subjected to HPLC on a μBondapak C_{18} Radial-Pak column and were eluted with a 0–55% linear acetonitrile gradient containing 0.2% TFA which was increasing in acetonitrile concentration at 10%/h. Flow-rate was 1 ml/min.

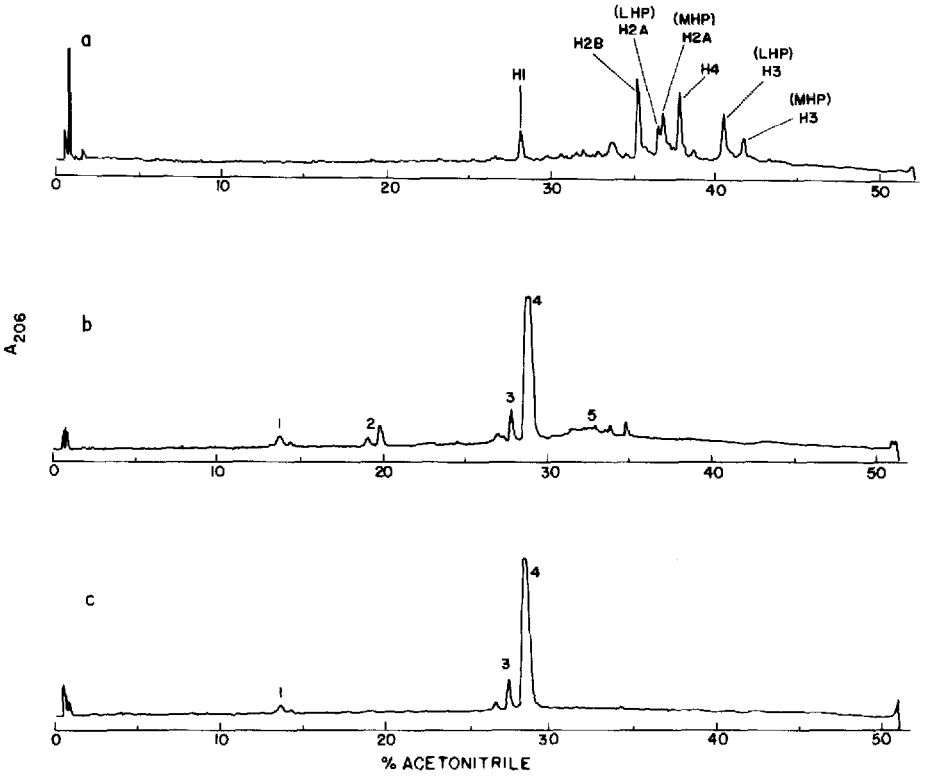

Fig. 2. Fractionation of sulfuric acid-soluble and perchloric acid-soluble chromatin proteins using a μBondapak CN column. Proteins were eluted for 5 h with a 0–50% linear acetonitrile gradient containing 0.2% TFA, flow-rate 1 ml/min. The gradient was generated by mixing water–0.2% TFA with water–acetonitrile (50:50) containing 0.2% TFA. (a) Whole histones, extracted with sulfuric acid from the chromatin of blended CHO cells. (b) H1–H1^0–HMG proteins, extracted with perchloric acid from the chromatin of blended CHO cells. (c) H1–H1^0–HMG proteins, extracted with perchloric acid from the chromatin of blended CHO nuclei.

baseline was produced at low acetonitrile concentrations (Fig. 2a). The oscillations probably result from some complex phenomenon involving volume contraction, which occurs when these solvents are mixed, and perhaps some incomplete solvent mixing as well, which in turn disturbs the equilibration of TFA between the mobile and stationary phases (TFA is responsible for most of the 206 nm absorbancy of the solvent). As a result of these observations, all of the experiments in this report were performed using premixed water–acetonitrile (50:50, v/v) containing 0.2% TFA as the organic solvent for generating acetonitrile gradients.

HPLC of H1, H1^0, and HMG proteins on a CN column

Histone H1, histone H1^0, and HMG proteins can be extracted as a group from chromatin with 5% perchloric acid and recovered by precipitation with trichloroacetic acid. When such a mixture was prepared from blended whole-cell chromatin and subjected to HPLC on a μBondapak CN column with a 0–50% acetonitrile gradient containing 0.2% TFA, five groups of proteins were eluted (Fig. 2b). When

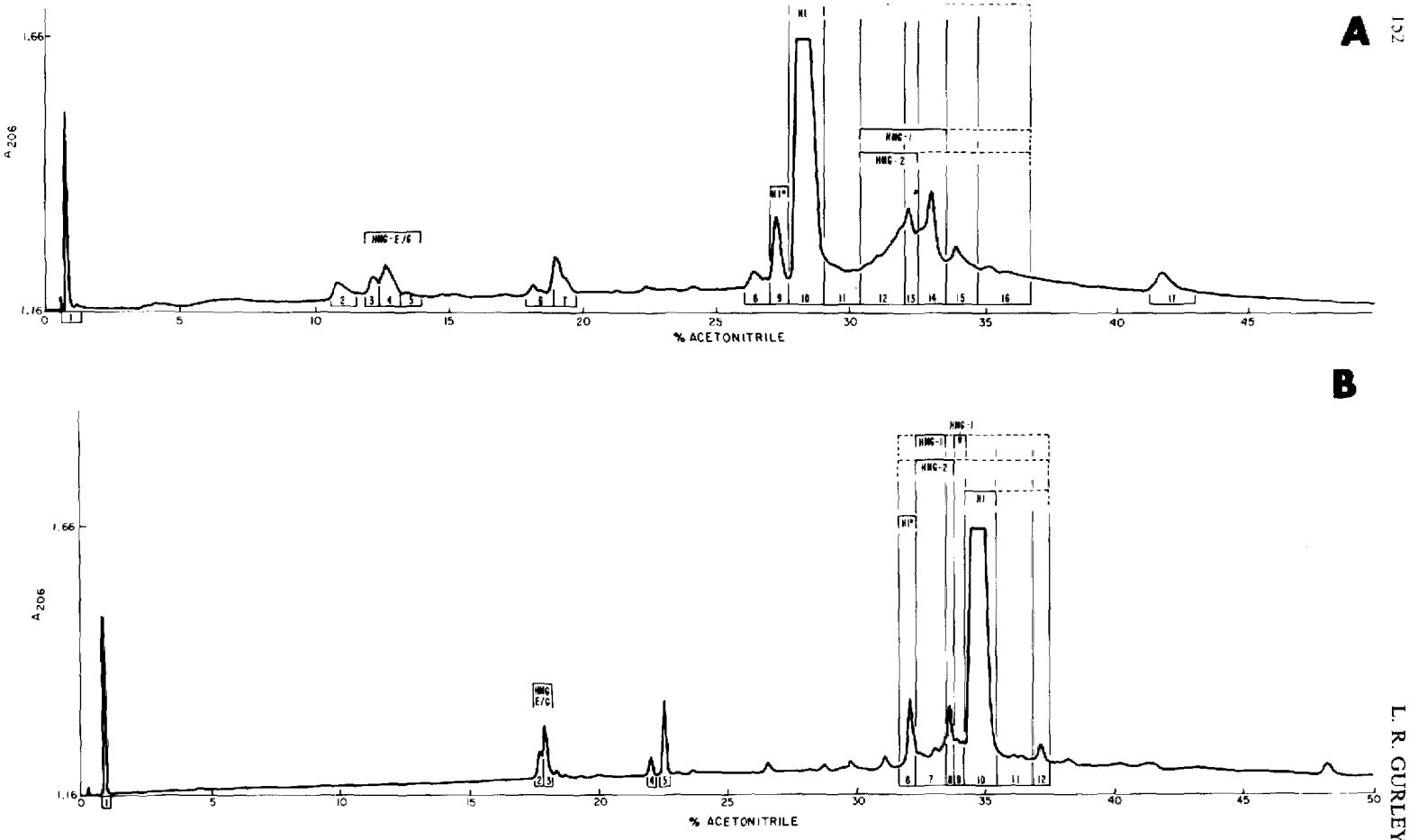

Fig. 3. Fractionation of perchloric acid-soluble proteins (H1, $H1^0$, HMG) on μBondapak CN and C_{18} columns. Proteins were eluted for 5 h with a 0–50% linear acetonitrile gradient containing 0.2% TFA. (A) μBondapak CN steel column, eluted at 1 ml/min; (B) μBondapak C_{18} Radial-Pak column, eluted at 2 ml/min. Each numbered peak was collected and freeze-dried for electrophoretic analysis.

the mixture was prepared from the chromatin of blended nuclei (nuclei prepared using the detergents Nonidet P-40 and sodium deoxycholate[10]), fewer proteins were eluted, *i.e.*, groups 2 and 5 were missing (Fig. 2c). Comparison of these chromatograms with those of whole histones (Fig. 2a) indicated that peak 4 was H1. The other proteins remained to be identified.

To identify the proteins in Fig. 2b, a preparative quantity (700 μg) of the mixture was subjected to HPLC (Fig. 3a). The effluent of each peak was freeze-dried and subjected to acid–urea gel electrophoresis for identification (Fig. 4). The identity of these peaks is marked on Fig. 3a. These proteins were found to be eluted in the following order of increasing acetonitrile concentration: HMG-E/G, an unidentified protein of high-mobility, $H1^0$, H1, HMG-2, HMG-1.

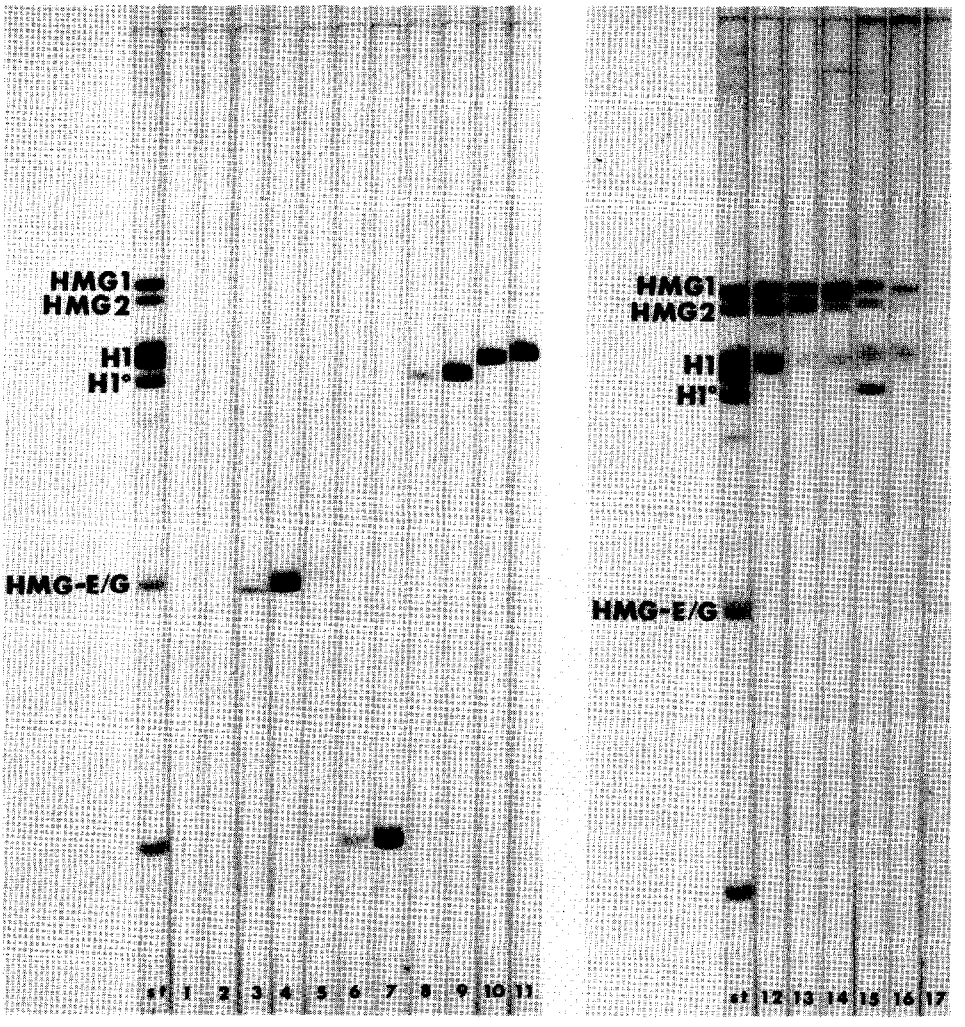

Fig. 4. Acid–urea polyacrylamide gel electrophoresis of the HPLC fractions eluted from the μBondapak CN steel column in Fig. 3A. Each numbered HPLC peak collected in Fig. 3A is shown along with a standard (st) sample of the preparation loaded onto the column.

The HMG-E/G proteins were fractionated into three peaks by HPLC, each peak with a slightly different electrophoretic mobility on acid–urea gels (Fig. 4). In contrast, the unidentified high-mobility protein was fractionated into two peaks by HPLC, but both peaks had the same electrophoretic mobility. The $H1^0$ and H1 peaks were eluted close together but were clearly resolved (Fig. 3a). Acid–urea gel electrophoresis indicated they were free from contamination with one another (Fig. 4). In this gel system, they also appeared to be homogeneous. Therefore, the μBondapak CN column system appeared to be a good system for the preparation of H1 and $H1^0$, as well as HMG-E/G.

The peaks eluted by 29–36% acetonitrile were complex (Fig. 3a). Electrophoresis indicated that a trailing shoulder of H1 was eluted in this region, contaminating the HMG-1 and HMG-2 proteins (Fig. 4). The HMG-1 and HMG-2 proteins were

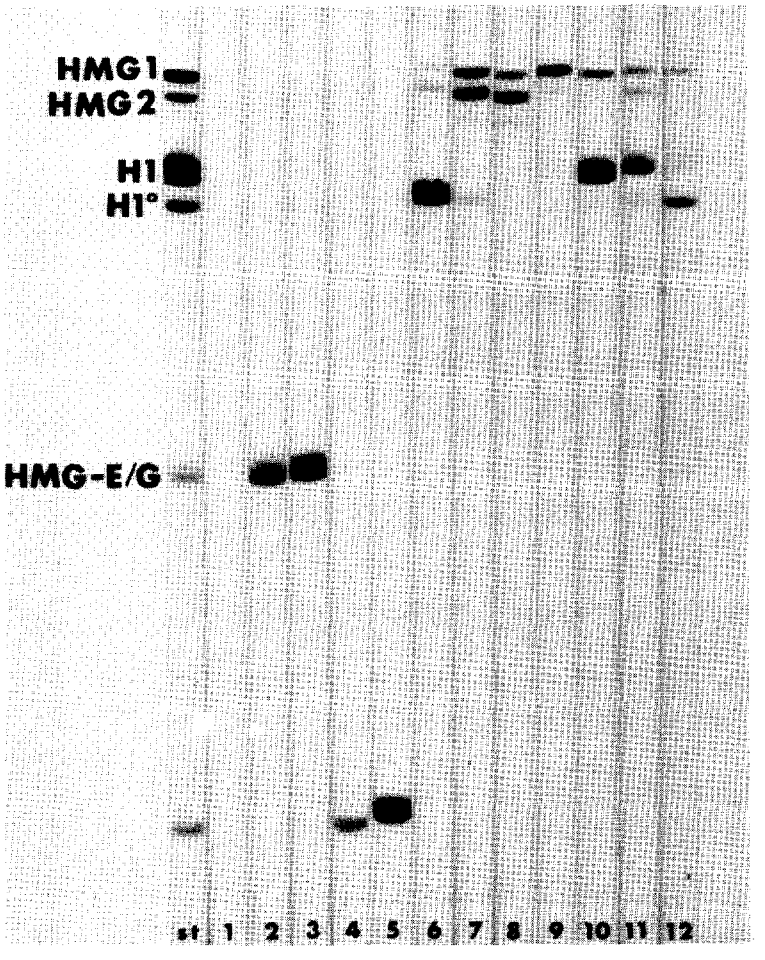

Fig. 5. Acid–urea polyacrylamide gel electrophoresis of the HPLC fractions eluted from the μBondapak C_{18} Radial-Pak column in Fig. 3B. Each numbered HPLC peak collected in Fig. 3B is shown, along with a standard (st) sample of the preparation loaded onto the column.

also not resolved (gels 12–14, Fig. 4). This appeared to be caused by leading shoulders on the HMG-1 and HMG-2 peaks which caused overlapping of these peaks with each other and with the trailing H1 shoulder (Fig. 3A). It is not clear at this time whether this complexity is caused by interactions between HMG-1, HMG-2, and H1, or due to some other phenomenon, such as multisite binding of these proteins. Further work will be required to find a method to resolve the HMG-1 and HMG-2 proteins.

HPLC of H1, H1^0, and HMG proteins on a C_{18} column

Preparative quantities (700 μg) of the H1, H1^0, and HMG mixture were also subjected to HPLC on a μBondapak C_{18} Radial-Pak column to see if this packing would resolve the HMG-1 and HMG-2 proteins (Fig. 3b). As expected from our earlier studies[5–7], the H1 was eluted at a higher acetonitrile concentration from the C_{18} packing (Fig. 3b) than from the CN packing (Fig. 3a). Acid–urea gel electrophoresis indicated that the H1^0 was also eluted at a higher acetonitrile concentration

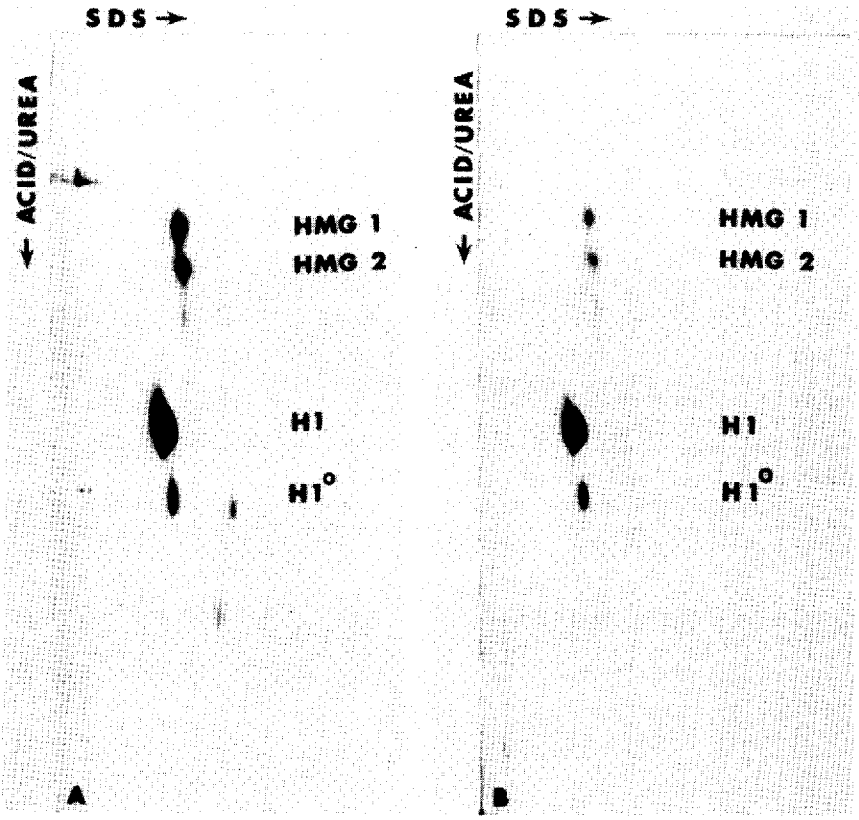

Fig. 6. Two-dimensional polyacrylamide gel electrophoresis of perchloric acid-soluble (H1, H1^0, HMG) chromatin proteins. The proteins were first separated in acid–urea cylindrical gels (top to bottom). The cylindrical gels were then placed on a Tris–glycine–SDS slab gel and again subjected to electrophoresis (left to right). (A) H1–H1^0 HMG proteins extracted from the chromatin of blended CHO cells. (B) H1 H1^0–HMG proteins extracted from the chromatin of blended CHO nuclei.

(Fig. 5). More importantly, the H1 and $H1^0$ were separated much further on the C_{18} column (Fig. 3b), a feature which will be used to advantage later in this report. Curiously, while the retention times of H1 and $H1^0$ on the C_{18} column were increased over their retention times on the CN column, the retention times of the HMG-1 and HMG-2 proteins were approximately the same on the two columns (compare Figs. 3a and 3b). This causes the $H1^0$ and H1 to be eluted simultaneously with the HMG-1 and HMG-2 proteins. Since the complex overlapping of the HMG-1 and HMG-2 proteins occurred on the C_{18} column (gels 6 and 7, Fig. 5), as well as on the CN column, it is clear that the CN column is the better system for fractionating H1 and $H1^0$.

$H1^0$ contaminants

Acid–urea gel electrophoresis of the $H1^0$ and H1 peaks, eluted from the μBondapak CN column (gel 9, Fig. 4), had indicated that these HPLC peaks were homogeneous. However, it was felt that this was not sufficient evidence to demonstrate the purity of these fractions. For example, when perchloric acid-extracted chromatin proteins are first subjected to acid–urea gel electrophoresis and these gels are then subjected to SDS gel electrophoresis in a second dimension, the $H1^0$ band is found to contain a contaminating lower-molecular-weight protein (Fig. 6a). Thus, it was felt that the purity of the H1 and $H1^0$ peaks from the HPLC should be further examined. SDS gel electrophoresis of the H1 and $H1^0$ peaks indicated that the H1 was homogeneous by this criterion (Fig. 7), as well as by acid–urea gel electrophoresis (Fig. 4, gel 10). On the other hand, $H1^0$ was found to contain two contaminants (Fig. 7). Contaminant "a" has an electrophoretic mobility in SDS gels which is similar to H1 (Fig. 7). Since H1 and $H1^0$ are eluted from the CN column very close together,

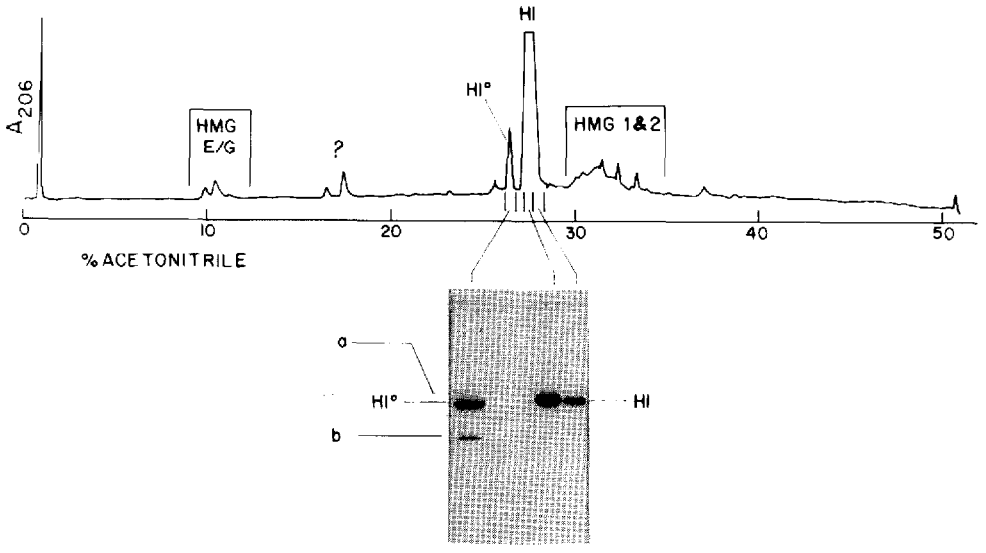

Fig. 7. SDS gel electrophoresis of H1 and $H1^0$ histones eluted from a μBondapak CN column. Contaminating proteins in the $H1^0$ peak are denoted by a and b. Perchloric acid-soluble chromatin proteins (548 μg) from CHO cells were subjected to HPLC. The histones and HMG proteins were eluted at 1 ml/min for 5 h with a 0–50% linear acetonitrile gradient containing 0.2% TFA.

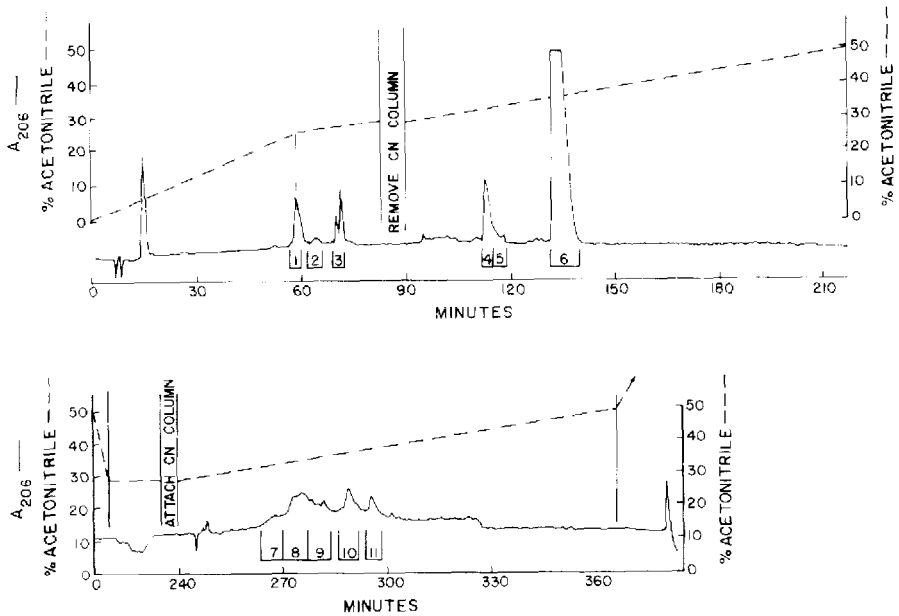

Fig. 8. Fractionation of perchloric acid-soluble proteins (H1, H1^0, HMG) using a tandem array of μBondapak CN and C_{18} columns. The HPLC components were initially connected in the following order: pumps, injector, μBondapak CN steel column, μBondapak C_{18} Radial-Pak cartridge contained in a Z-module, UV detector. Proteins (730 μg in 200 μl) were loaded onto the CN column and eluted through both CN and C_{18} columns at 1 ml/min for 60 min with a 0 25% linear acetonitrile gradient containing 0.2% TFA. Elution was then continued using a 25–29% linear gradient for 24 min. Solvent flow was stopped and the CN column was removed (6 min). Elution of the C_{18} column was then continued at 1 ml/min using a 29–50% linear gradient for 126 min. The solvent pumping through the C_{18} column was then returned to 29% acetonitrile (4 min) and the C_{18} column was equilibrated at 29% acetonitrile for 15 min. The solvent flow was stopped and the CN column (which contained 29% acetonitrile solvent) was placed in its original position ahead of the C_{18} column (5 min). The proteins remaining on the CN column were then eluted through both CN and C_{18} columns at 1 ml/min with a 29 50% linear gradient in 126 min. The columns were finally purged with 100% acetonitrile containing 0.2% TFA. Each numbered peak was collected and freeze-dried for electrophoretic analysis.

it would not be surprising to find such contamination. However, this probably is not the case, since no H1 was observed in a previous H1^0 peak examined by acid–urea gel electrophoresis (Fig. 4, gel 9). Contaminant "b" is probably the same one as that observed in the two-dimensional gel electrophoresis of perchloric acid-extracted proteins (Fig. 6a). This contaminant can be greatly reduced if nuclei are prepared prior to perchloric acid extraction (Fig. 6b). However, the use of nuclei isolation procedures prior to extraction is often unacceptable to those researchers interested in keeping the HMG proteins in their preparations, because HMG-1 and HMG-2 are easily lost during nuclei isolation (Figs. 2c and 6b). Thus, the search for a method to obtain pure H1^0 preparations by HPLC was continued.

HPLC of H1, H1^0 and HMG proteins on tandem μBondapak columns

In Fig. 3, four properties of the μBondapak reversed-phase systems are observed which might be used to achieve purification of H1^0: (1) the H1^0 and H1 peaks

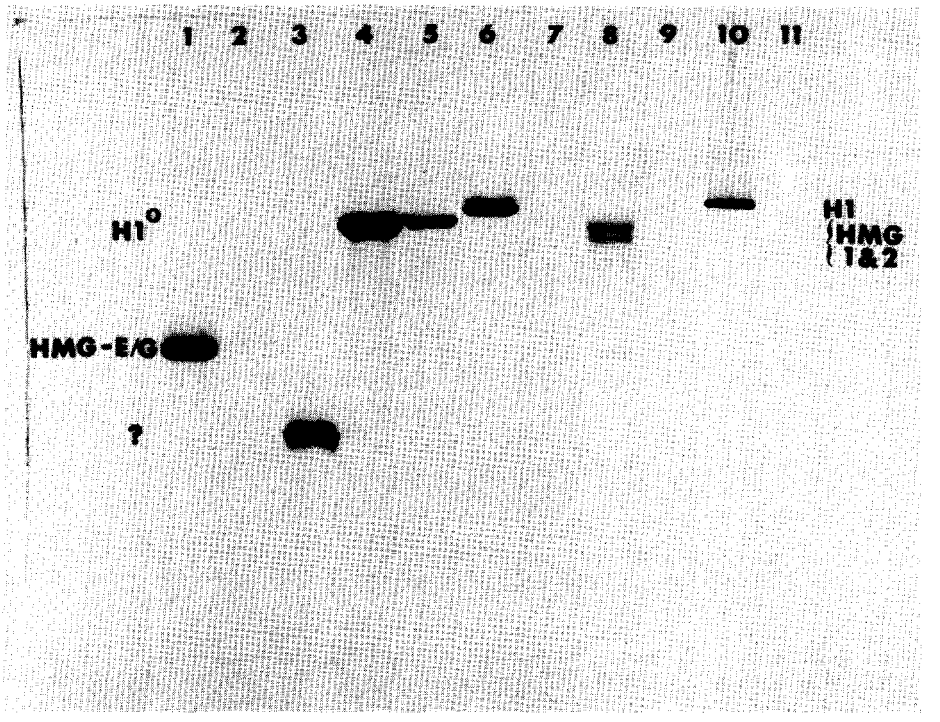

Fig. 9. SDS gel electrophoresis of the HPLC fractions, eluted from the tandem array of μBondapak CN and C_{18} columns in Fig. 8. Each numbered HPLC peak collected in Fig. 8 is shown in a similarly numbered lane in the SDS slab gel.

are separated from the HMG proteins on the CN column; (2) $H1^0$ and H1 are better separated from one another on the C_{18} column; (3) the H1 and $H1^0$ peaks are eluted from the CN column at less than 29% acetonitrile, but at more than 29% acetonitrile from the C_{18} column; (4) the nonhistone HMG-1 and HMG-2 proteins are eluted from the C_{18} and CN columns at approximately the same concentration of acetonitrile; perhaps some other nonhistone proteins are eluted at this same concentration also.

From these properties, it was predicted that if a C_{18} column was attached to the effluent end of a CN column and a gradient of acetonitrile from 0–29% was passed through both of them, the $H1^0$ and H1 would be eluted from the CN column and re-adsorbed on the C_{18} column, but the HMG-1 and HMG-2 proteins would remain adsorbed on the CN columns. This was found to be the case. In Fig. 8, three peaks are seen to be eluted from the C_{18} column attached to the end of the CN column. Peaks 1 and 3 were identified by SDS gel electrophoresis as HMG-E/G and the unknown high-mobility protein, respectively (Fig. 9, gels 1 and 3). Since these proteins were found to be homogeneous in both acid–urea gels and in SDS gels (Fig. 9), whether they were obtained from CN or C_{18} columns, it is concluded that they are pure preparations of these proteins.

From the properties listed above, it was also predicted that if the CN column was removed from the gradient at 29% acetonitrile, the HMG-1 and HMG-2 proteins

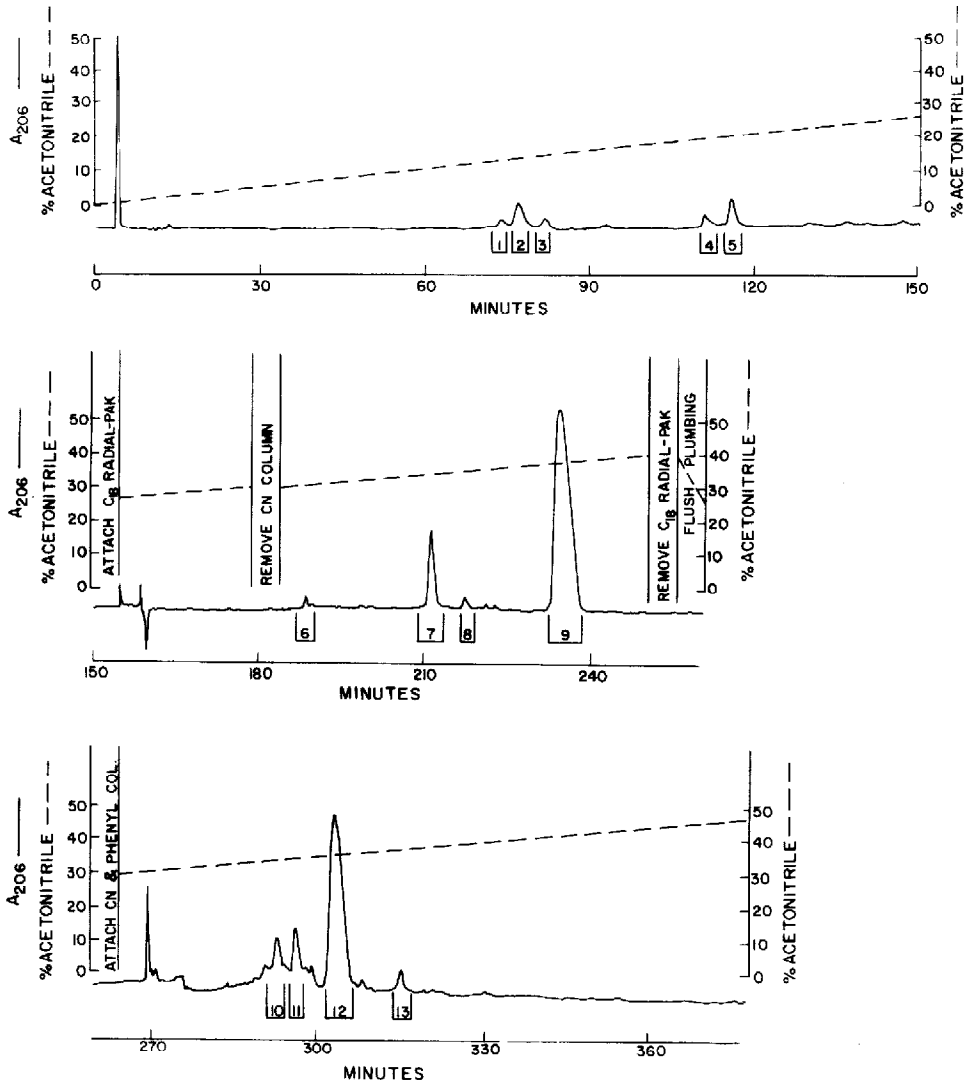

Fig. 10. Fractionation of perchloric acid-soluble proteins (H1, H1^0, HMG) using an alternating tandem system of μBondapak CN, C_{18} and phenyl columns. The HPLC components were initially connected in the following order: pumps, injector, μBondapak CN column, UV detector. Proteins (800 μg in 200 μl) were loaded onto the CN column and eluted at 1 ml/min for 150 min with a 0–25% linear acetonitrile gradient containing 0.2% TFA. Solvent flow was stopped and a μBondapak C_{18} Radial-Pak cartridge, contained in a Z-module and equilibrated with 25% acetonitrile, was inserted between the CN column and the UV detector (5 min). Elution of the CN column onto the C_{18} column was then continued at 1 ml/min using a 25–29% linear gradient for 24 min. Solvent flow was stopped and the CN column was removed (5 min). The C_{18} Radial-Pak was then eluted at 1 ml/min with a 29–40% linear gradient for 66 min. Solvent flow was stopped, the C_{18} Radial-Pak was removed, and the solvent lines and injector were flushed with 29% acetonitrile (10 min). The μBondapak CN column containing the HMG-1 and HMG-2 proteins was re-attached to the HPLC system, followed by a μBondapak phenyl steel column, which had been equilibrated with 29% acetonitrile–0.2% TFA (5 min). The CN column was then eluted through the phenyl column with a 29–50% linear acetonitrile gradient at 1 ml/min for 126 min. Each numbered peak was collected and lyophilized to a dry powder for electrophoretic analysis.

would be removed from the system, thus allowing elution of $H1^0$ and H1 from the C_{18} column with improved resolution at higher acetonitrile concentrations without superimposing them on the HMG proteins, as they were in Fig. 3b. This was also found to be the case (Fig. 8). The $H1^0$ and H1 peaks were eluted at 33% and 36% acetonitrile, respectively, and both were found to be pure by SDS gel electrophoresis (Fig. 9, gels 4–6). The more slowly migrating H1 band in lane 6 of Fig. 9 is the minor H1 variant found in CHO cells[17]. It is not resolved by HPLC on μBondapak columns. Thus, this method can be used to prepare $H1^0$ and total pure H1 proteins.

After elution of the $H1^0$ and H1 proteins, the C_{18} column was re-equilibrated to 29% acetonitrile and attached to the effluent end of the CN column containing the HMG-1 and HMG-2 proteins. With a linear gradient of acetonitrile from 29–50%, the HMG-1 and HMG-2 proteins were eluted together from the CN and C_{18} columns in peak number 8 (Fig. 9, gel 8). The trailing shoulder of H1 which is eluted from the CN column at acetonitrile concentrations greater than 29% was eluted from the C_{18} column in peak number 10 (Fig. 9, gel 10).

Because the CN column gives a better fractionation of the HMG-E/G proteins and the unknown high-mobility proteins, a slightly different manipulation of the CN and C_{18} columns was used to take advantage of this. First, the H1–$H1^0$–HMG mixture was loaded on a CN column (there was no C_{18} column in the system). Elution of the CN column with a 0–25% acetonitrile gradient produced three HMG-E/G peaks and two unknown high-mobility peaks (Fig. 10). Then, a C_{18} column was

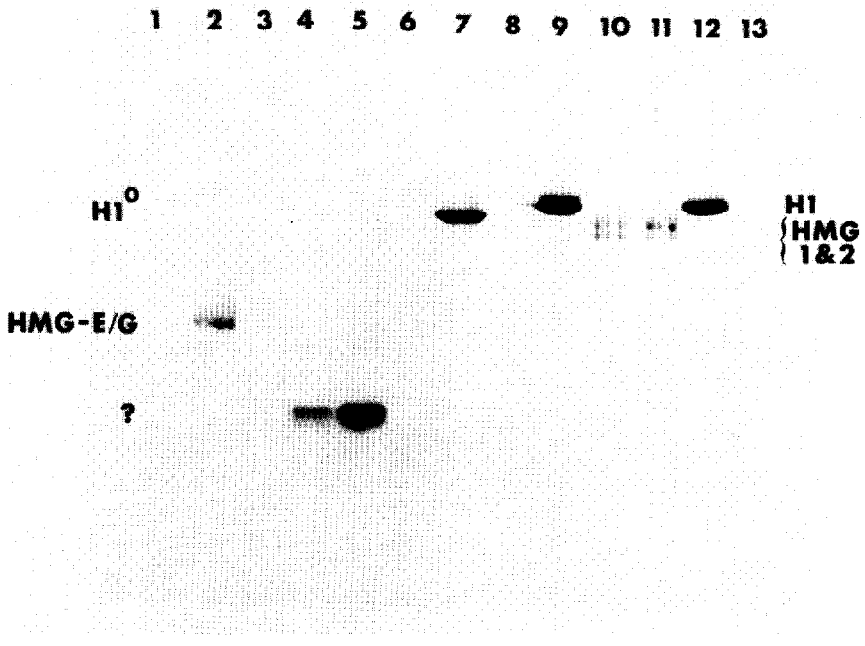

Fig. 11. SDS gel electrophoresis of the HPLC fractions eluted from the μBondapak CN, C_{18}, and phenyl columns in Fig. 10. Each numbered HPLC peak collected in Fig. 10 is shown in a similarly numbered lane in the SDS slab gel.

attached to the effluent end of the CN column, and the gradient was continued from 25–29% acetonitrile, which eluted the $H1^0$ and H1 from the CN column and caused re-adsorption of these histones on the C_{18} column. The CN column containing the HMG-1 and HMG-2 proteins was then removed and the $H1^0$ and H1 were eluted from the C_{18} column with a 29–40% acetonitrile gradient (Fig. 10). This procedure produced pure preparations of H1 and $H1^0$ (Fig. 11) which are easily recovered by lyophilization.

We had shown that neither the μBondapak CN nor C_{18} columns produced satisfactory fractionation of HMG-1 from HMG-2 (Figs. 3–5, 10, and 11). Since phenyl reversed-phase columns sometimes produce different chromatograms from CN and C_{18} reversed-phase columns, we wanted to see if the μBondapak phenyl column would resolve the CHO HMG-1 and HMG-2. Previous work with this phenyl column had been discouraging because of a high 206-nm-absorbing solvent background[7]. However, detailed study indicated that a low-background window existed between 20% and 50% acetonitrile if a shallow gradient was used. Therefore, we equilibrated a μBondapak phenyl column at 29% acetonitrile and attached it to the effluent end of the CN column (Fig. 10) which contained the HMG-1 and HMG-2

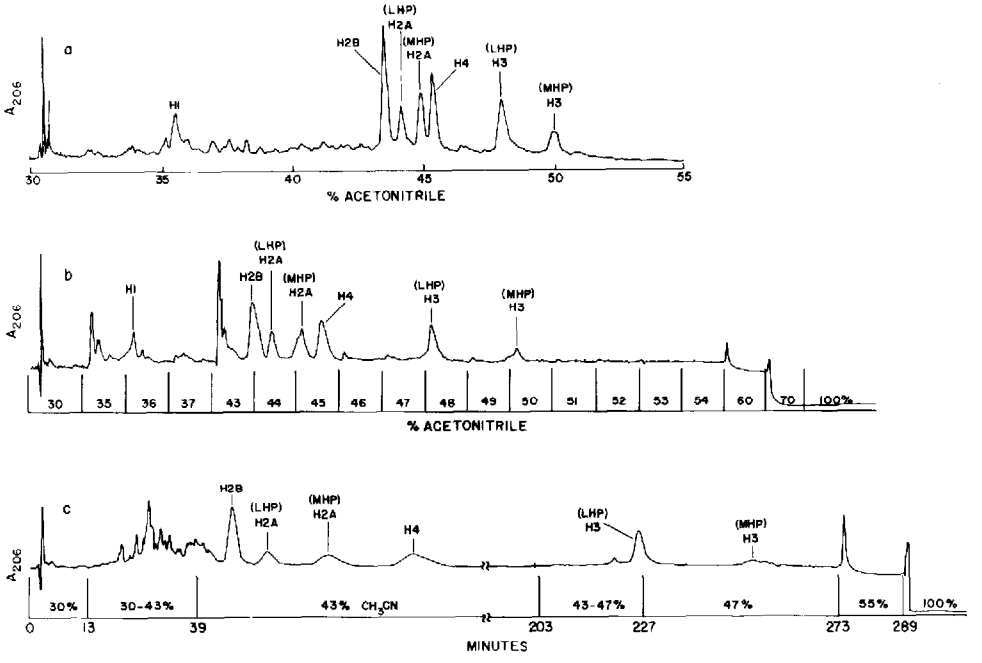

Fig. 12. Fractionation of sulfuric acid-soluble proteins, extracted from blended CHO cell chromatin, using a μBondapak C_{18} Radial-Pak column and various eluting solvents containing acetonitrile, water and 0.3% TFA. Whole CHO histones (933 μg in 200 μl) were loaded onto the column equilibrated with 30% acetonitrile–0.3% TFA. (a) The histones were eluted at 2 ml/min for 150 min with a 30–55% linear gradient increasing in acetonitrile concentration at a rate of 10%/h. The gradient was generated by mixing 100% water–0.3% TFA with 100% acetonitrile–0.3% TFA. (b) The histones were eluted at 2 ml/min using 10-min steps of acetonitrile at the percentages indicated in the figure. (c) The histones were chromatographed at 2 ml/min with an isocratic elution at 30% acetonitrile, followed by a 30–43% linear gradient, followed by an isocratic elution at 43% acetonitrile, followed by a 43 47% linear gradient, followed by isocratic elutions at 47%, 55%, and 100%, using the time schedule shown in the figure.

proteins. The two columns were eluted with a gradient from 29–50% acetonitrile. Two HMG peaks were eluted (Fig. 10, peaks 10 and 11). Peak 10 was enriched in HMG-2 and peak 11 was enriched in HMG-1, but both were contaminated with each other (Fig. 11). The trailing shoulder of H1 from the CN column was eluted as a pure fraction in peak 12 (Fig. 11). Thus, we have not yet found conditions for the preparative HPLC of HMG-1 and HMG-2 on the series of μBondapak reversed-phase columns.

Preparation of H2A and H3 variants on μBondapak C_{18} Radial-Pak columns

We have previously shown that steel columns loaded with μBondapak C_{18} packing would not resolve the more hydrophobic (MHP) variant of H2A from histone H4[5]. While steel columns loaded with μBondapak CN packing would resolve these two histones, they would not completely separate the (MHP)H2A from the less hydrophobic (LHP) variant of H2A (Fig. 2a)[6]. Recently, work with Radial-Pak cartridges[7] demonstrated that the HPLC of histones on μBondapak C_{18} Radial-Pak columns greatly improved the resolution of these histones if either 0.2% TFA (Fig. 1) or 0.3% TFA (Fig. 12a) were present in the acetonitrile gradient. However, 0.1% TFA was not sufficient to accomplish this[7].

While the chromatogram in Fig. 12a is quite acceptable for many studies, total resolution is required if HPLC is to be used for the preparation of pure proteins. It was felt that the total resolution of the four histones [*e.g.* H2B, (LHP)H2A, (MHP)H2A and H4] might be accomplished by eluting them individually at their specific acetonitrile dissociation concentrations. The chromatogram in Fig. 12b shows that elution of the column with increasing steps of acetonitrile (each containing 0.3% TFA) resulted in H1 being eluted at 36% acetonitrile, H2B at 43%, (LHP)H2A at 44%, (MHP)H2A at 44%, and H4 at 45%. (LHP)H3 and (MHP)H3 were eluted at 48% and 50%, respectively. Resolution of the peaks appears to be complete. The

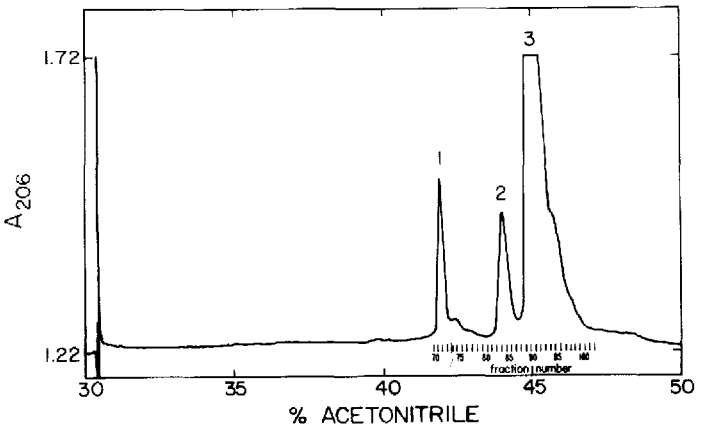

Fig. 13. Fractionation of Bio-Gel P-100-purified *Drosophila melanogaster* H2A/D2 using a μBondapak C_{18} Radial-Pak column. H2A/D2 (870 μg in 261 μl of water containing 0.2% TFA) was loaded onto a column equilibrated with 30% acetonitrile–0.2% TFA, and then eluted at 2 ml/min for 120 min with a 30–50% linear acetonitrile gradient containing 0.2% TFA. Fractions of the effluent were collected at 1-min intervals (2 ml/fraction) and freeze-dried. Fractions 70–102 were subjected to electrophoretic analysis.

shoulders on the peaks probably are the result of the step changes in acetonitrile concentration.

While isocratic elution of total histones from reversed-phase columns had previously not been successful in our laboratory, the resolution of (LHP)H2A and MHP(H2A) during the 44% acetonitrile step in Fig. 12b suggested that isocratic elution might have a limited application in the preparative HPLC of the H2B–H2A–H4 group. Indeed, it was found that the four histones could be completely resolved if the gradient was stopped at 43% acetonitrile and isocratic elution at this concentration was continued (Fig. 12c). It was also found that the (MHP)H3 variant could be eluted by isocratic elution at 47% acetonitrile (Fig. 12c). These experiments demonstrate that HPLC can be used for the preparative isolation of these nucleosomal histone variants.

Preparation of Drosophila H2A and D2 histones

Because mammalian histone H2A variants could be fractionated by HPLC, it was thought that perhaps this method could be used to fractionate the *Drosophila*-specific histone D2 from *Drosophila* H2A. The preparation of these two histones has

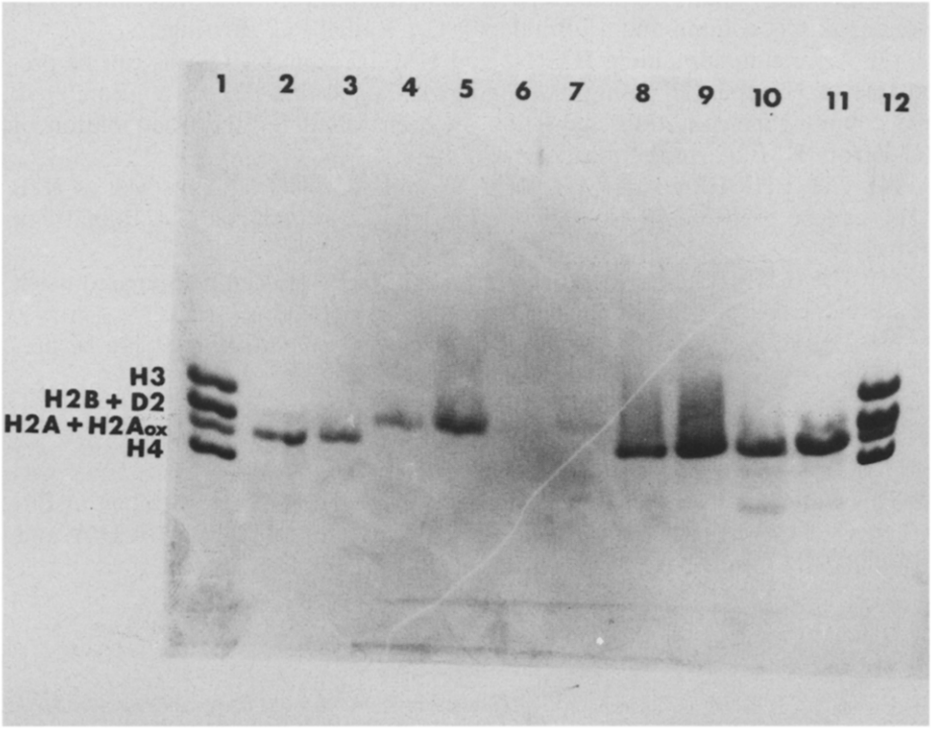

Fig. 14. SDS gel electrophoresis of the HPLC fractions of *Drosophila melanogastor* H2A/D2, eluted from the μBondapak C_{18} Radial-Pak column in Fig. 13. Lanes 1 and 12 in the slab gel are *D. melanogastor* embryo whole histones; lanes 2 and 11 are unfractionated H2A/D2; lane 3 is $H2A_{ox}$ from peak 1 (Fraction 72 in Fig. 13); lanes 4 and 5 are D2 from peak 2 (fraction 85); lanes 6 and 7 are from the valley between peaks 2 and 3 (fraction 87); lane 8 is H2A from peak 3 (fraction 91); lanes 9 and 10 are also H2A from peak 3 (fraction 92).

been a problem, since they are isolated together when separated from the other *Drosophila* histones by Bio-Gel P-100 column chromatography[11].

When a preparation of Bio-Gel P-100-purified *D. melanogaster* H2A/D2 was subjected to HPLC on the μBondapak C_{18} Radial-Pak column using a 30–50% acetonitrile linear gradient containing 0.2% TFA, three peaks were obtained (Fig. 13). The first and third peaks were identified as H2A by SDS gel electrophoresis (Fig. 14), the second peak was identified as D2. The first H2A peak has been identified as the oxidized form of H2A using Triton DF-16 gel electrophoresis[11]. It remains to be determined whether this oxidation of H2A occurs during histone preparation from the *Drosophila* embryos or during the HPLC procedure itself. SDS gel electrophoresis (Fig. 14) indicates that HPLC can be used to obtain pure preparations of these three proteins.

CONCLUSIONS

The following conclusions have been made:

(1) Pure preparations of CHO HMG-E/G, an unidentified high-mobility protein, and H1 can be obtained by HPLC using a μBondapak CN column;

(2) Pure $H1^0$ and H1 can be prepared using a tandem column system involving a μBondapak CN column and a μBondapak C_{18} Radial-Pak cartridge;

(3) A fraction containing HMG-1 and HMG-2 from CHO cells can be prepared free of H1 and $H1^0$ using a μBondapak C_{18} Radial-Pak or a μBondapak phenyl column, but conditions have not yet been found for the fractionation of HMG-1 from HMG-2 on the μBondapak columns.

(4) The (LHP)H2A and (MHP)H2A variants of CHO H2A, as well as H2B and H4, can be prepared by isocratic elution from a μBondapak C_{18} Radial-Pak cartridge.

(5) The (LHP)H3 and (MHP)H3 variants of CHO H3 can be prepared using either μBondapak CN or μBondapak C_{18} Radial-Pak columns.

(6) The H2A, D2, and oxidized H2A from *Drosophila* embryos can be prepared using the μBondapak C_{18} Radial-Pak cartridge.

ACKNOWLEDGEMENTS

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Erratum

High-performance liquid chromatography of chromatin histones, by L. R. Gurley, D. A. Prentice, J. G. Valdez and W. D. Spall, *J. Chromatogr.*, 266 (1983) 609–627.

p. 610, line 4 of the section *High-performance liquid chromatography* should read: "packing of octadecylsilane bonded to silica which had not been end-capped; (2)".

p. 626, line 16 should read: "Because of this interference of detection by TFA, efforts should now be directed".