Journal of Chromatography, 559 (1991) 431–443
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 493

High-performance capillary electrophoresis of histones

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ABSTRACT

A high-performance capillary electrophoresis (HPCE) system was developed for the fractionation of histones. This system involves electroinjection of the sample and electrophoresis in 0.1 *M* phosphate buffer (pH 2.5) in a 35 cm × 50 μm I.D. coated capllary. Electrophoresis was accomplished in 9 min, separating a whole histone preparation into its components in the following order of decreasing mobility: (MHP) H3, H1 (major variant), H1 (minor variant), (LHP) H3, (MHP) H2A (major variant), (LHP) H2A, H4, H2B and (MHP) H2A (minor variant), where MHP is the more hydrophobic component and LHP is the less hydrophobic component. This order of separation is very different from that found in acid–urea polyacrylamide gel electrophoresis and in reversed-phase high-performance liquid chromatography and, thus, brings the histone biochemist a new dimension for the qualitative analysis of histone samples.

INTRODUCTION

The separation of histones by zone electrophoresis in a starch gel matrix was first introduced by Neelin and Connell over 30 years ago [1]. Since that time, the gel electrophoresis of histones, particularly in polyacrylamide gels, has been widely practiced [2–12] because it is inexpensive to implement and simple to operate [13]. On the other hand, the gel preparation is tedious, the electrophoresis is time consuming and quantification involves staining, destaining and scanning desitometry, which is labor intensive, time consuming, and semi-quantitative [13].

The advent of high-performance capillary electrophoresis (HPCE) [14,15] potentially offers a new opportunity to raise the electrophoretic fractionation and analysis of histones to a performance level superior to that of gel electrophoresis. The advantages of HPCE should include high resolution, rapid analysis time, on-line detection and computer-based quantification. Most important, HPCE has the potential for the analysis of very small samples in the femtomole range [15]. In our laboratory, the ability to perform analyses on such small samples is attractive because it would facilitate the analysis of histones from the small numbers of cells acquired by flow cytometry cell sorting. For these reasons, we have initiated a program to develop HPCE methods to fractionate nucleoproteins. This paper describes the development of the separation of histones by zone electrophoresis in free solution using low-pH buffers in coated silica capillaries.

0021-9673/91/$03.50

EXPERIMENTAL

Cel cultures and histone preparation

Chinese hamster cells (line CHO) were grown exponentially in suspension culture as described by Tobey *et al.* [16]. Chromatin was prepared from whole-cell homogenates as described by Gurley *et al.* [17]. Histones were extracted from the chromatin with 0.2 *M* sulfuric acid, recovered by acetone precipitation and stored as a lyophilized powder at −70°C until used for HPCE [17].

To prepare purified histones for high-performance liquid chromatography (HPLC), 3×10^8 nuclei were prepared from CHO cells using the detergents Nonidet P-40 and sodium deoxycholate in hypotonic solution as described by Gurley *et al.* [18]. The nuclei were then dissolved for 2 h in 1 ml of reagent containing 6 *M* guanidine · HCl, 0.2% trifluoroacetic acid (TFA), 0.155 *M* NaCl, 0.026 *M* HCl and 0.025% dithiothreitol according to the method of Jackson and Gurley [19]. Undissolved residual nuclei were sedimented by centrifugation at 8000 *g* for 10 min. The supernatant fluid containing soluble DNA and proteins was decanted into 9 ml of water containing 0.2% TFA. The residual undissolved nuclei were dissolved in 1 ml of dissolving reagent for 1 h and then centrifuged as before. Only a trace of nuclear material remained undissolved. The supernatant fluid was decanted into the water–0.2% TFA containing the first decantate. The DNA in the solution was precipitated overnight at 4°C due to the acidic conditions of the solution. The DNA was sedimented by centrifugation at 8000 *g* for 2 h. The 11 ml of supernatant fluid, containing the total complement of proteins from the cell nuclei, was stored at −70°C. When used for HPLC, this solution was thawed and centrifuged for 2 h at 8000 *g* to remove any residual DNA clouding the sample.

HPLC of histones

The guanidine-soluble DNA, salts and other chemical reagents in the dissolved nuclei samples were separated from the nuclear proteins by size-exclusion HPLC. This was accomplished as described by Jackson and Gurley [19] using a Waters HPLC system equipped with a Bio-Sil TSK guard column followed by a Bio-Sil TSK size-exclusion column (Bio-Rad Labs). The sample was injected into the guard column and was eluted through the two columns with water–0.2% TFA at a flow-rate of 0.5 ml/min. The guard column adsorbed any soluble DNA remaining in the sample and the proteins were separated from the salts and other chemical reagents by size-exclusion HPLC on the Bio-Sil TSK column [19]. The effluent protein fraction was either lyophilized to a dry powder and stored at −70°C for future use or it was injected directly into a reversed-phase μBondapak CN HPLC column (Waters Assoc.) [20] which adsorbed the proteins on the top of the column [21].

The histone proteins were fractionated and purified by reversed-phase HPLC on the μBondapak CN column using gradient elution with acetonitrile containing 0.2% of TFA as described by Gurley *et al.* [22]. This column fractionated the histones into the five basic types, H1, H2A, H2B, H3 and H4, and the H2A and H3 histones were subfractionated into two variants of each, the less hydrophobic (LHP) H2A and (LHP) H3 and the more hydrophobic (MHP) H2A and (MHP) H3 [20–22]. Each of these seven individual histone fractions was collected and either lyophilized to dryness or evaporated to dryness while centrifuging in a vacuum chamber (Speed-Vac)

and stored at − 70°C [22] until used to identify the individual histones fractionated by HPCE.

HPCE of histones

HPCE was performed using a Model HPE-100 high-performance electrophoresis system manufactured by Bio-Rad Labs. Experiments were performed using 20 cm × 25 μm I.D., 35 cm × 50 μm I.D., and 50 cm × 50 μm I.D. capillary tubes whose inside surfaces were chemically modified to produce a patented hydrophilic coating. Coatings of this nature have been reported by several laboratories to eliminate electroendoosmosis and protein adsorption [23–25]. Cathode, anode and capillary buffers were 0.1 *M* sodium phosphate (pH 2.5) (Bio-Rad Labs.) unless specified otherwise. Detection of the separated proteins traversing the capillary was accomplished by in-tube monitoring of the peptide bond absorbance at 200 nm with a spectrophotometer setting of 0.005 a.u.f.s. To evaluate the peformance of this instrument, a standard mixture of nine polypeptides was subjected to electrophoresis. This mixture contained bradykinin, angiotensin II, α-MSH, TRH, LHRH, [2–5] leucine enkephalin, bombesin, methionine enkephalin and oxytocin (Bio-Rad Labs).

Samples were prepared for HPCE by dissolving lyophilized histone powder in water containing 0.2% of TFA at a concentration of 0.1 μg/μl. Poly-L-lysine hydrobromide, Type VI, molecular mass 13 000 (Sigma) was added to some of these solutions at a concentration of 0.05 μg/μl to serve as an internal mobility marker during electrophoresis. These samples were loaded into the capillaries by electroinjection [26]. To accomplish this, the cathode chamber and capillary were filled with 0.1 *M* phosphate buffer and the anode chamber was filled with water. Then 5 μl of sample were injected into the loading chamber at the anode end between the capillary and the water. Power was applied to the capillary at 10 kV (constant voltage) and *ca.* 38 μA for 10 s. This actively carries the cations (including the positively charged protonated proteins) into the capillary. Under these low ionic strength sample conditions, the proteins are concentrated and stacked in the capillary during this loading step [27]. The excess of sample in the loading chamber and the water in the anode chamber were then replaced with 0.1 *M* phosphate buffer and electrophoresis was performed by applying power to the capillary at 10 kV (constant voltage) and *ca.* 41 μA. Electrophoresis normally took 10 min using the 35 cm × 50 μm I.D. coated capillary.

RESULTS

20 cm × 25 μm I.D. capillary

The performance of the HPE-100 high-performance electrophoresis instrument is typically evaluated by subjecting a mixture of nine polypeptides to zone electrophoresis in a 20 cm × 25 μm I.D. coated capillary using 0.1 *M* phosphate buffer (pH 2.5). In our laboratory repetitive electrophoresis of this standard produced fractions whose peak heights varied by only 3.0% (relative standard deviation) and whose mobilities varied by 7.2%. This high performance led us to choose these operating conditions for our first attempts to separate histones by HPCE. We found that when whole histones were dissolved in water containing 0.2% of TFA (as they are when recovered from an HPLC column) we could electroinject them into the capillary in 10 s at 10 kV. However, electrophoresis at 10 kV produced a single peak of unresolved

proteins. By decreasing the electrophoresis voltage we found that this single peak contained the various histone fractions which had mobilities ranging over 2 min at 4 kV, but they could not be resolved under these conditions (Fig. 1A).

It was thought that perhaps protein–protein interactions in the capillary might cause a degradation of resolution. To prevent this, NaCl was added to the electrophoresis buffer. Preliminary experiments indicated that when NaCl concentrations greater than 0.12 *M* was used, the electrophoresis was inhibited owing to the elevated conductivity of the buffer. However, when 0.12 *M* NaCl was used, histone electrophoresis was accomplished. Histone resolution at 4 kV was better in the presence of 0.12 *M* NaCl (Fig. 1B) than it was without NaCl (Fig. 1A). However, it was not adequate for analytical use.

50 cm × 50 μm I.D. Capillary

Another possible cause of the poor resolution might be protein interactions with the capillary walls. To reduce the magnitude of this effect we performed the electrophoresis in a larger diameter capillary having the dimensions of 50 cm × 50 μm I.D. After electroinjection at 10 kV, the histones were subjected to electrophoresis

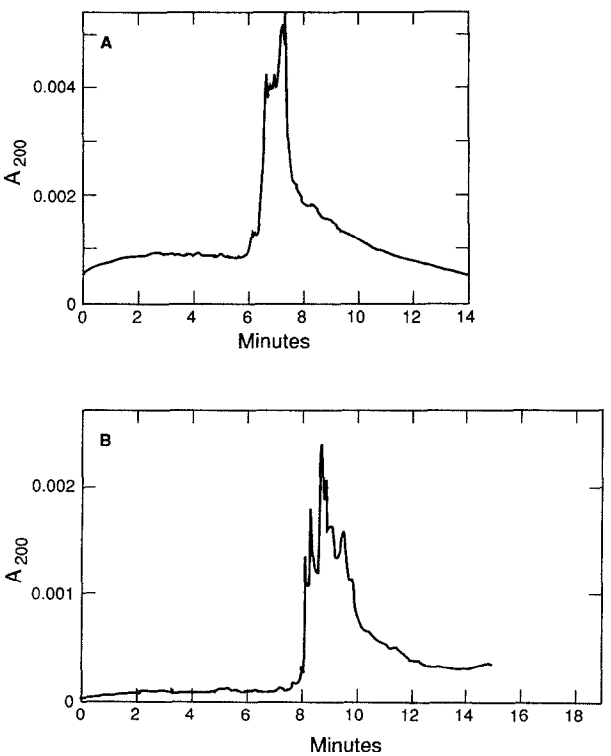

Fig. 1. HPCE of whole histones in a 20 cm × 25 μm I.D. coated capillary. Samples were electroinjected for 10 s at 10 kV. Electrophoresis was performed at 4 kV in 0.1 *M* phosphate buffer (pH 2.5) (A) without NaCl or (B) containing 0.12 *M* NaCl. Resolution was monitored at 200 nm with a detector response time of 1 s and a chart speed of 1 cm/min.

at four different voltages (4, 6, 8 and 10 kV). We found that the resolution of the histones was far superior in this 50 μm I.D. capillary (Fig. 2A) than in the 25 μm I.D. capillary (Fig. 1A). Reducing the electrophoresis voltage from 10 to 6 kV appeared to improve the resolution. However, close inspection indicated that although the peaks are spread further apart at 6 kV than at 10 kV, the longer electrophoresis time permitted diffusion to spread the bands so that no significant increase in resolution was accomplished at the lower voltages.

NaCl was added to the electrophoresis buffer in the 50 cm × 50 μm I.D. capillary. There was a small increase in the resolution when the NaCl concentration was increased from 0 *M* (Fig. 2A) to 0.12 *M* (Fig. 2B). The most noticeable effect of NaCl was with the largest fraction, which was latter identified as H2B. When the

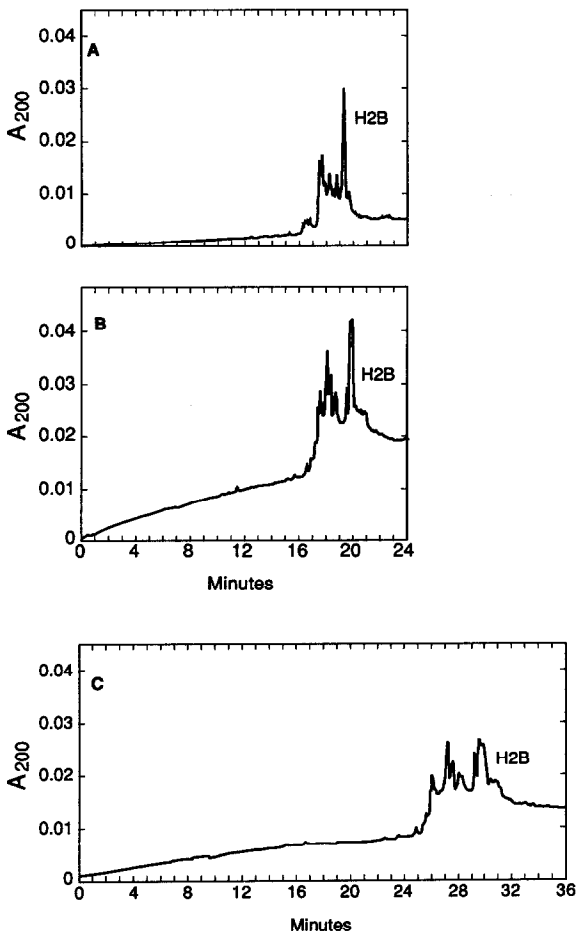

Fig. 2. HPCE of whole histones in a 50 cm × 50 μm I.D. coated capillary. Samples were electroinjected for 10 s at 10 kV. Electrophoresis was performed at 10 kV in 0.1 *M* phosphate buffer (pH 2.5) (A) without NaCl and (B) containing 0.12 *M* NaCl, and (C) at 7 kV in 0.1 *M* phosphate buffer (pH 2.5) containing 0.12 *M* NaCl. Resolution was monitored as in Fig. 1.

electrophoresis voltage was reduced from 10 to 7 kV in the presence of 0.12 *M* NaCl, the H2B peak was observed to undergo partial resolution into more fractions (Fig. 2C). Unfortunately, the longer electrophoresis time at 7 kV permits too much band broadening due to diffusion, thus destroying the overall resolution of the whole sample (Fig. 2C).

35 cm × 50 μm I.D. capillary

Since diffusion appeared to be a significant factor in the degradation of the electropherogram, we used a shorter capillary (35 cm × 50 μm I.D.) to reduce the time of the run. With the shorter capillary, the histones migration took less than 8 min at 10 kV (Fig. 3B) whereas with the 50-cm capillary it took 15–17 min (Fig. 3A). This capillary size appeared to give the best resolution of the three examined, so we adopted this capillary to investigate other operational parameters.

pH of the electrophoresis buffer

All the previous experiments were performed at pH 2.5. To determine if this pH was the optimum condition for resolving the histones, the pH was varied in the 35 cm × 50 μm I.D. capillary from 2.0 to pH 5.0 (Fig. 4). We found that histones moved the fastest at pH 2.0 (Fig. 4A) and the slowest at pH 3.5 (Fig. 4E). The best resolution was obtained at pH 2.5 (Fig. 4B). Runs at higher pH produced greater overlapping frac-

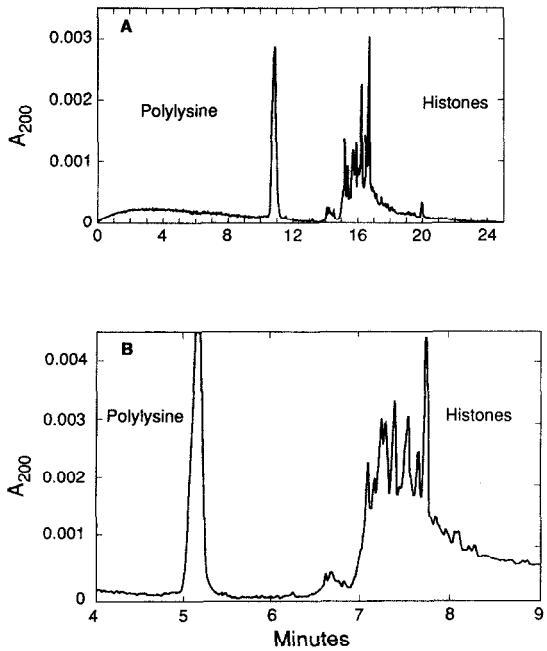

Fig. 3. HPCE of whole histones in a 35 cm × 50 μm I.D. coated capillary. Samples were electroinjected for 10 s at 10 kV and electrophoresed at 10 kV. A comparison is shown between electrophoresis in (A) a 50 cm × 50 μm I.D. and (B) a 35 cm × 50 μm I.D. capillary. Resolution was monitored as in Fig. 1 except that the chart speed of (A) 1 cm/min was increased to (B) 6 cm/min in order to facilitate visualization of the resolution of the closely spaced bands in the 35 cm × 50 μm I.D. capillary.

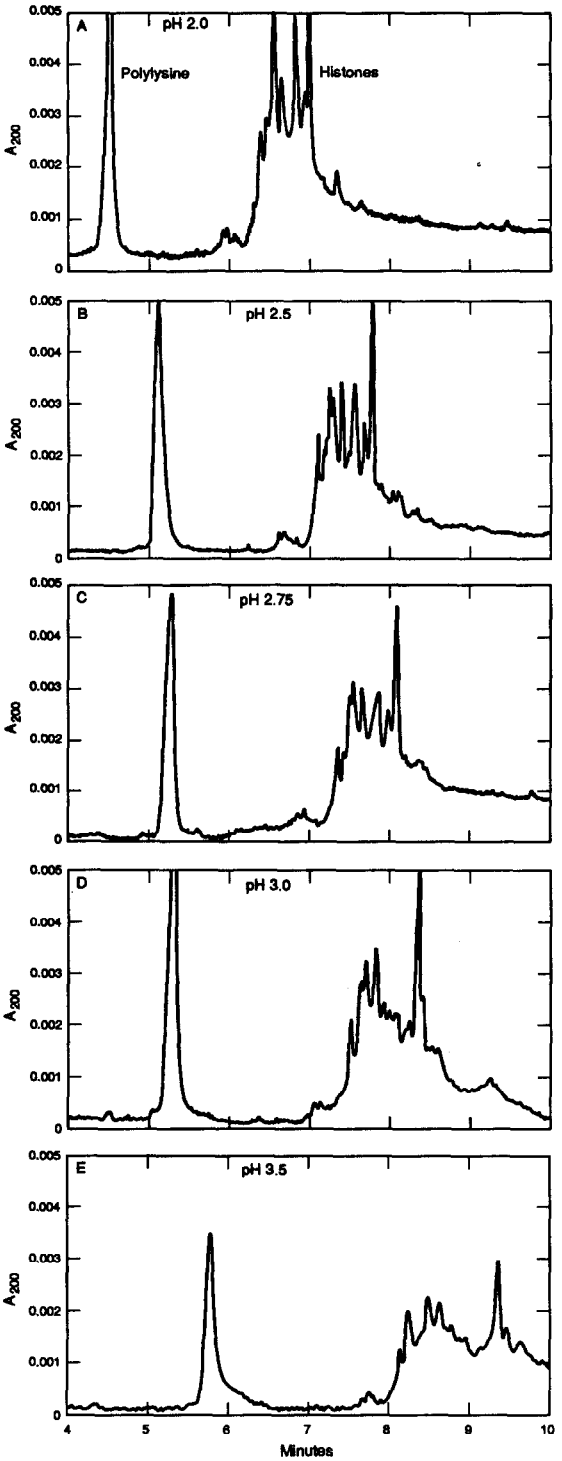

Fig. 4. HPCE of whole histones in a 35 cm × 50 μm I.D. coated capillary at various pH values. All samples were electroinjected for 10 s at 10 kV. Electrophoresis was performed in 0.1 *M* phosphate buffer at pH (A) 2.0, (B) 2.5, (C) 2.75, (D) 3.0 and (E) 3.5. Resolution was monitored at a chart speed of 3 cm/min.

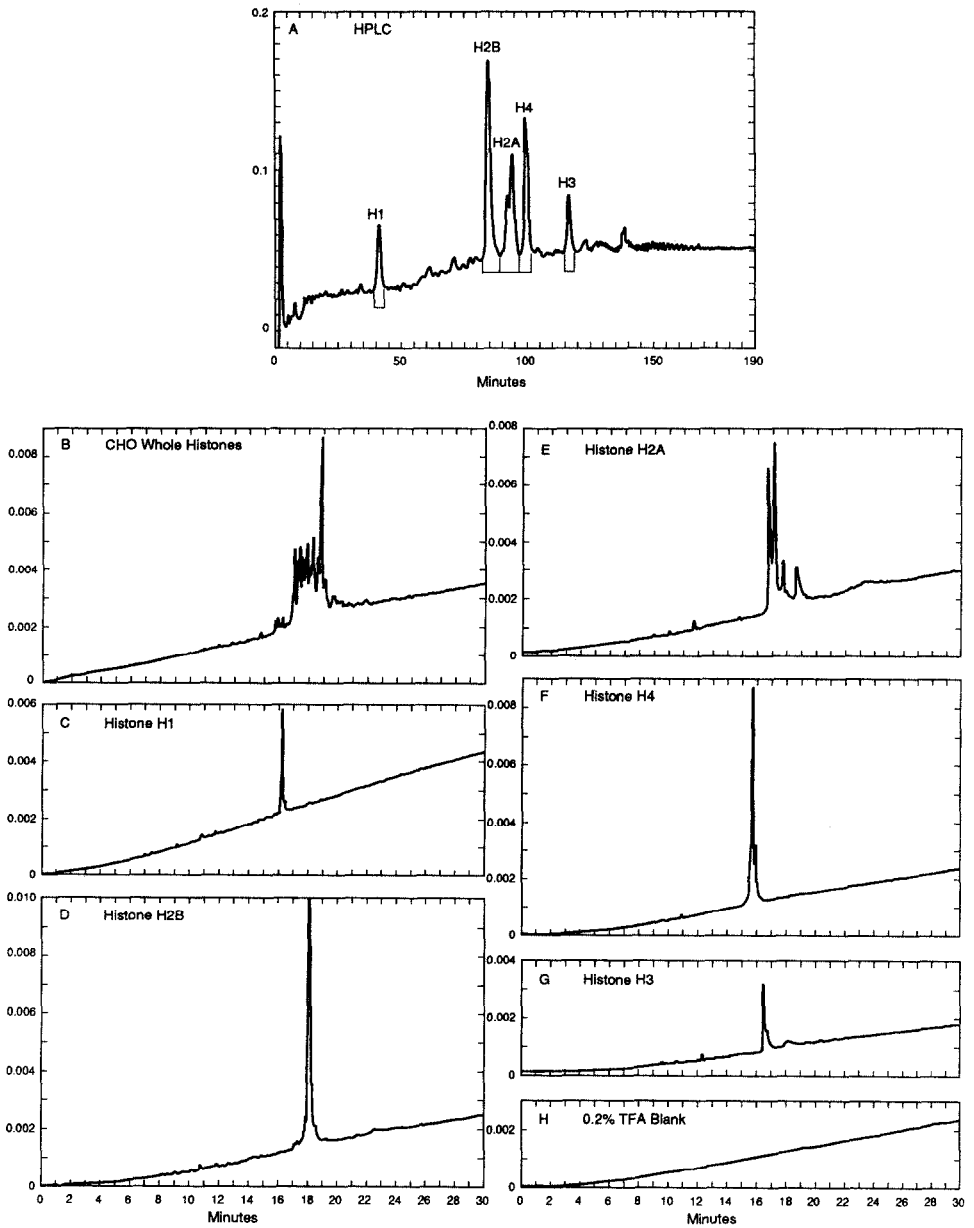

Fig. 5. HPCE of histone fractions in a 50 cm × 50 μm I.D. coated capillary. (A) The histones of CHO nuclei were fractionated into the five histone classes (H1, H2B, H2A, H4 and H3) by reversed-phase HPLC. (B) Whole unfractionated histones, (C) H1, (D) H2B, (E) H2A, (F) H4, (G) H3 and (H) a 0.2% TFA blank solution in which these histones were dissolved were subjected to HPCE in 0.1 *M* phosphate buffer (pH 2.5) at 10 kV. Resolution was monitored as in Fig. 1.

tions and above pH 3.5 the electropherogram became a broad, unresolved smear. Therefore, pH 2.5 appears to be the optimum condition for this set of proteins.

Identification of the histone fractions

Our first attempt to identify the individual histone fractions in the electropherograms was made while using the 50 cm × 50 μm I.D. capillary. Purified histone fractions (H1, H2A, H2B, H3 and H4) were obtained from CHO nuclei by HPLC (Fig. 5A). Each of these five fractions was subjected to electrophoresis at pH 2.5 (Fig. 5, C–G) and compared with a whole histone preparation (Fig. 5B) and a water–0.2% TFA blank, the solvent in which the samples were dissolved (Fig. 5H). The fractions were found to be pure by HPCE, H1 and H2B migrating as a single peak, H2A migrating as four variants which are known to exist for this histone, histone H4 migrating as a single peak with a presumptive acetylated form following it and H3 migrating as a single peak with its presumptive acetylated form following it. The fractions had migration times varying from 16 to 20 min (Fig. 5, C–G). The whole histones had migration times from 17 to 20 min. We pointed out at the beginning of the paper that there was a 7.2% variability in the mobility of standards. This variability made it impossible to use the mobility of the individual histone fractions to identify the histone fractions in the whole histone electropherogram in Fig. 5B.

In some experiments, polylysine was added to samples as an internal standard in the hope that this mobility variation could be normalized as a ratio of the mobility of the fractions to that of polylysine (Fig. 3). However, this did not reduce the variability sufficiently to provide unequivocal assignments based on the individual fraction mobilities.

This problem was overcome by adding individual histone fractions to the whole histone sample. Seven individual histone fractions were isolated by HPLC (Fig. 6A). Each of these fractions was mixed with a separate sample of whole histone containing polylysine and loaded into a 35 cm × 50 μm I.D. capillary for 10 s at 10 kV. The samples were then subjected to electrophoresis at 10 kV for 10 min. Each fraction in the electropherogram of the whole histone sample was located by the increase in the fraction's peak height (Fig. 6, B–H). From these data, the order of mobility of each histone fraction was determined (Fig. 7). From the fastest to the slowest, they are (MHP) H3, H1 (major variant), H1 (minor variant), (LHP) H3, (MHP) H2A (major variant), (LHP) H2A, H4, H2B, (MHP) H2A (minor variant). Under these conditions, all the fractions move past the detector within 1 min. Two unidentified fractions are detectable in the whole histone electropherogram, one between the (LHP) H2A and H4 and the other between the H4 and H2B (Fig. 7). The whole histone preparations also contain a cluster of unidentified high-mobility minor components which precede the histones at 7 min and a cluster of unidentified low-mobility minor components which follow the H2B at 8 min (Fig. 7).

DISCUSSION

These experiments represent the development of the first application of HPCE to histone analysis. The optimum conditions that we have found to date involve sample electroinjection for 10 s at 10 kV and electrophoresis at 10 kV under acid conditions at pH 2.5 in 0.1 *M* phosphate buffer in a 35 cm × 50 μm I.D. coated

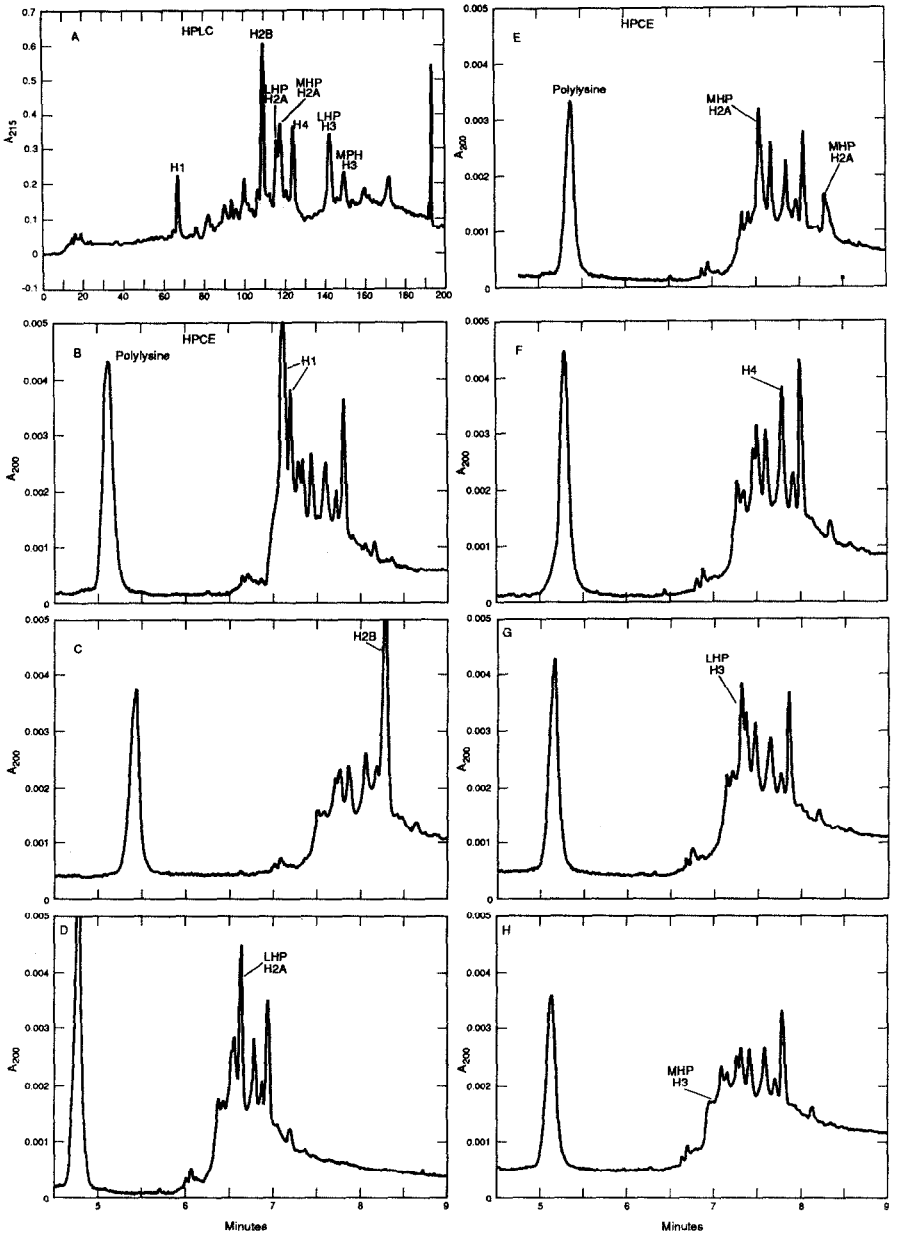

Fig. 6. HPCE of whole histones spiked with individual histone fractions using a 35 cm × 50 μm I.D. coated capillary. (A) The histones of CHO nuclei were fractionated into the five histone classes (H1, H2B, H2A, H4 and H3) and two variants each of H2A and H3, which are indicated as the less hydrophobic (LHP) and more hydrophobic (MHP) forms. (B–H) A sample of each of these fractions was evaporated to dryness and added to a separate whole histone preparation together with polylysine (internal standard) and subjected to HPCE in 0.1 *M* phosphate buffer (pH 2.5) at 10 kV. Resolution was monitored at a chart speed of 6 cm/min. (B) Histone H1 (C) H2B, (D) LHP-H2A, (E) MHP-H2A, (F) H4, (G) LHP-H3 and (H) MHP-H3 were identified in the whole histone electropherograms by the increase in the peak height of an individual component.

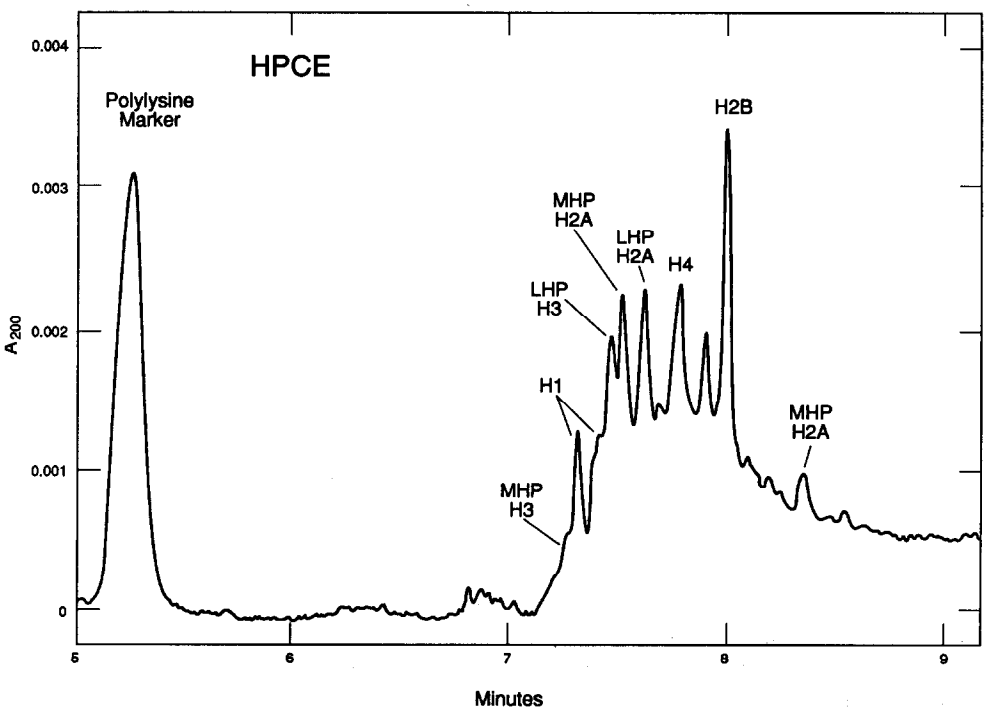

Fig. 7. Assignment of histone fractions obtained by HPCE of a whole histone sample. HPCE was performed in a 35 cm × 50 μm I.D. coated capillary. The sample was dissolved in 0.2% TFA and electroinjected for 10 s at 10 kV. Electrophoresis was performed at 10 kV in 0.1 *M* phosphate buffer (pH 2.5). Resolution was monitored at 200 nm with a detector response time of 1 s and a chart speed of 6 cm/min. Polylysine was added to the sample as an internal mobility standard.

capillary. Some of the criteria for high performance are met by this system. Small sample volumes (5 μl) can be used and electrophoresis is accomplished in a short time (9 min). In-capillary detection by UV absorption at 200 nm is possible provided that the sample is concentrated enough (0.1 μg/μl). This permits the use of computers to perform on-line data analysis of the electropherograms. This procedure also fulfills our need to be able to perform analysis on the histones from a small number of cells, such as those acquired by flow cytometry cell sorting. For example, a 5-μl sample at 0.1 μg/μl is the amount of histones we can obtain from 55 000 CHO cells.

The order in which the histone fractions migrate in HPCE is very different from that found in acid–urea polyacrylamide gel electrophoresis [8,12] or in reversed-phase HPLC (Figs. 5A and 6A). For this reason, HPCE brings the histone biochemist a new dimension for the qualitative analysis of histone samples. It also provides a quick method of quality control for evaluating the purity of isolated individual histone fractions. For example, we demonstrated that histone fractions isolated by reversed-phase HPLC were pure (Fig. 5).

The apparent greater amount of H2B compared with the other histone fractions (Fig. 7) may reflect heterogeneity in that band. When we added 0.12 *M* NaCl to the electrophoresis buffer and decreased the voltage to 7 kV, this fraction broadened

and appeared to develop a slower moving component as a trailing shoulder (Fig. 2C). Further work will be required te determine what this extra component is. Also, two other unknown components were observed in whole histone preparations, one preceding and the other following H4. These also need to be identified. As they are readily separated by HPCE, we shall be able to use this method as an assay for them. This will enable us to search chromatograms for their presence and then verify their purity during isolation.

The system has two problems that need to be dealt with further: narrowly spaced bands and broadly diffused bands. These problems are not restricted to the HPCE of histones, as we have also encountered them with the HPCE of plasma proteins. As we do not encounter these problems when performing HPCE on small peptide standards, they appear to be problems associated strictly with macromolecular proteins. These problems interfere most seriously during quantitative analysis. For example, at the beginning of the Results section we pointed out that we could obtain peak heights that varied by only 3.0% when using small peptide standards. This high precision has not been obtainable for histones or other proteins, however.

To improve the HPCE of histones, a system needs to be found that will separate the bands further apart in a shorter time. At present, all the histone fractions pass the detector within 1 min. The use of longer capillaries did not increase their resolution because it also increased the time taken to traverse the capillary, thus broadening the bands due to diffusion. However, the close bands would not be such a problem if the protein bands were narrow like those of small peptides. Hence the most serious problem to be solved is that of band broadening.

It may be that the broader protein bands result from incomplete stacking of the proteins during the electroinjection step. It has been suggested that protein–protein interactions may cause such an interference. Our experience with polylysine argues against this reasoning, however. We found that polylysine, having a molecular mass of 13 000, produced a broad band like a protein (Fig. 7). We would not expect this highly positively charged macromolecule to produce significant intermolecular interactions. An alternative suggestion is that the hydration shell around the proteins may be much greater than that around the small peptides, thus preventing the high degree of concentration from occurring during the stacking process. If this in the case, it may be difficult in the HPCE of proteins to produce the narrow bands observed with peptides and we shall be forced to find a system that produces bands spaced further apart in order to increase resoluton.

Although the HPCE of proteins has several shortcomings at this early stage of development, capillary electrophoresis still offers the potential for further development into an important high-performance analytical system for the histones. This work lays the groundwork and experience for further experiments to solve the resolution and quantification problems associated with the application of this new technology to protein biochemistry.

ACKNOWLEDGEMENTS

This work was performed under the auspices of the US Department of Energy's Office of Health and Environmental Research.

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