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ELECTROPHORESIS WITH TWO BUFFERS IN ONE DIMENSION IN THE ANALYSIS OF GLYCOSAMINOGLYCANS ON CELLULOSE ACETATE STRIPS

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SUMMARY

We have systematically examined the electrophoretic behavior of hyaluronic acid, desulfated chondroitin, heparan sulfate, and chondroitin sulfate with two different buffers under varying conditions of buffer concentration, electrophoresis time, and voltage. An anodal effect was observed with barium acetate buffer in which the glycosaminoglycans behaved as if they became positively charged as they neared the anode, whereas with monovalent buffers the migration was nearly linear with time. Such differences in behavior permitted us to develop a two-buffer monodirectional electrophoresis technique which offers some of the advantages of resolution seen with two-dimensional methods, yet retains the advantages of band comparison and quantitative analysis characteristic of one-dimensional electrophoresis.

INTRODUCTION

Despite the widespread use of electrophoresis on cellulose acetate as an analytical tool for glycosaminoglycans (GAGs) its usefulness in separating complex mixtures of these polyanions has been limited by the similarity in migration of several of the more common species. Solutions to this problem have been sought by modifying buffer systems or support media [1—4], by comparing two separate electrophoretic analyses under different buffer conditions [5, 6], or by combining the two analyses into a single two-dimensional electrophoresis [7, 8]. Cappelletti et al. [9] recently used the differential solubilities of GAGs in ethanol during electrophoresis to permit the simultaneous separation of at least eight species. Their technique appears to offer the greatest resolution of published methods, but the use of an ice-containing chamber in which the strips are submerged in chilled decane during electrophoresis may be too restrictive for many applications.

We have attempted to combine these various methods in a technique which

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would be convenient, reproducible, and permit the clean separation of the four major GAGs of embryonic tissue: hyaluronic acid, heparan sulfate, chondroitin sulfate and unsulfated chondroitin. Unsulfated chondroitin is of increasing interest in studies of GAG biosynthesis [10—12]; yet there is no published method for demonstrating it electrophoretically. The usual method of analysis of unsulfated chondroitin involves enzymatic degradation [13]. Our technique is analogous to two-dimensional electrophoresis in that it makes use of the properties of two buffers, but it is run in one dimension through the sequential applications of the buffers. This produces bands, rather than the diffuse and irregularly shaped spots obtained by two-dimensional techniques, and it also offers the convenience and analytical advantages of comparing several samples and standards on a single strip. In this paper we briefly present experiments which provide the rationale for this approach, and include some observations on a significant [14] anodal effect that should be of theoretical importance in the design of electrophoretic analyses.

MATERIALS AND METHODS

Reagents

NIH standards of heparan sulfate (HS), chondroitin-4-sulfate (C-4S), and chondroitin-6-sulfate (C-6S) were generous gifts of J.A. Cifonelli (University of Chicago). The other GAGs were from Sigma (St. Louis, MO, U.S.A.). Desulfated chondroitin (C-0S) was prepared from Sigma C-4S with acidic methanol [15]. Sepraphore III cellulose acetate strips (2.5 × 17 cm and 5.7 × 14.4 cm) and the electrophoresis chamber were from Gelman (Ann Arbor, MI, U.S.A.). Titan III cellulose acetate plates (6.0 × 7.6 cm) from Helena Laboratories (Beaumont, TX, U.S.A.) were tested successfully but not used for the work reported here.

Electrophoresis

Standard conditions are described; any variations are indicated in the text. The buffers were 0.05 *M* barium acetate adjusted to pH 5.8 with glacial acetic acid [1, 16] and 0.05 *M* formic acid adjusted to pH 3.0 with pyridine [17, 18]. Alcian blue staining solution was prepared by dissolving the dye (0.1%, w/v) in a washing solution, which consisted of 0.025 *M* sodium acetate (pH 5.8) and 0.05 *M* magnesium chloride diluted with an equal volume of ethanol [16].

(1) Dry cellulose acetate strips were marked with ink 2.5 cm from one end to indicate the future site of sample application and were then hydrated for at least 10 min in a one-tenth dilution of the formate—pyridine buffer.

(2) A buffer interface was prepared in order to stack the sample in a narrow band, by a technique based on that of Cappelletti et al. [9]. The hydrated strip was blotted on filter paper and immersed in full-strength (0.05 *M*) formate—pyridine buffer from the future anodal end of the strip up to about 1 cm in front of the site of sample application. Three rapid in and out immersions were sufficient to exchange the buffer. The strip was blotted again and placed in the electrophoresis tray.

(3) The samples (1—2 μl containing 0.1—5 μg of GAG) were applied im-

mediately on small chips of cellulose acetate strip, and the first electrophoresis was performed for 30 min at room temperature at 200 V (20 V/cm). Full-strength buffer was at the anode, but the one-tenth dilution was at the cathode to help maintain the interface during sample application.

(4) After electrophoresis the strips were immersed in 95% ethanol for 5 min to precipitate the GAGs and transferred to a solution of the second buffer (0.05 *M* barium acetate) and ethanol (1:3, v/v), for another 5 min.

(5) The strips were blotted, and the alcohol was allowed to evaporate until speckled dry areas began to appear (usually within 1 min). They were immediately blotted again between two filter papers which had been saturated with 0.05 *M* barium acetate but which had been pressed with a rubber roller to leave them only slightly damp. This last step completed the transfer into the second buffer. Direct immersion into the second buffer without prior precipitation was avoided in quantitative studies to prevent loss of sample.

(6) The second electrophoresis was carried out for 20 min at 200 V with 0.05 *M* barium acetate buffer at both the anode and cathode.

(7) The strips were immersed for 5 min in 95% ethanol and stained with Alcian blue for 30 min with continuous gentle agitation. They were then blotted and destained in three 10-min baths of washing solution with agitation [16].

RESULTS

Comparison of electrophoresis conditions

Two buffers which are widely used to separate GAGs are barium acetate [1, 5, 16] and formate–pyridine [5, 6, 17, 18]. Accordingly, we compared the migration of HA and C-6S with these buffers under different conditions (Fig. 1). With increasing molarity of either barium acetate (pH 5.8) or formate–pyridine (pH 3.0), the bands migrated closer together (Fig. 1a), but the

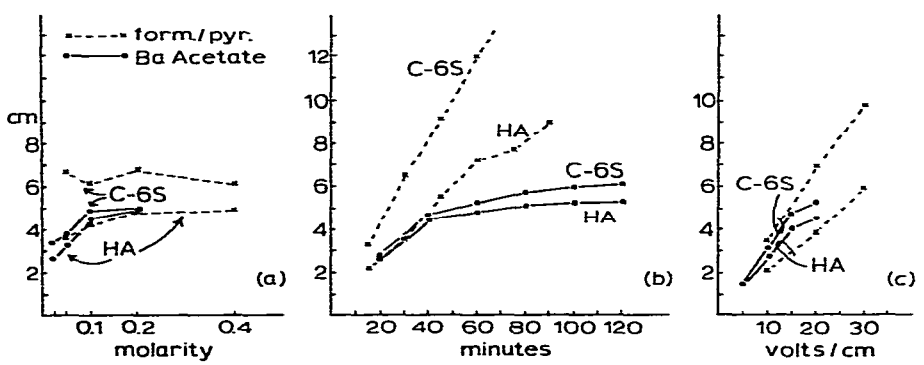

Fig. 1. Effects of varying buffer concentration, electrophoresis time, and voltage. Standard HA and C-6S (1 μg in 1 μl) were applied separately to cellulose acetate strips and electrophoresed under varying conditions. The GAGs were stained with Alcian blue and recorded for measurement by xerography. (a) Migration distance vs. buffer concentration at 20 V/cm for 30 min in formate–pyridine (pH 3.0) or 60 min in barium acetate (pH 5.8). (b) Migration distance vs. time at 20 V/cm in 0.05 *M* buffers. The C-6S in formate–pyridine buffer moved off the end of the strip at 12.5 cm. (c) Migration distance vs. voltage in 0.05 *M* buffers for 30 min in formate–pyridine and 60 min in barium acetate.

bands were also less diffuse at higher molarities. As the buffer molarities approached zero the GAGs were the major charged species and showed strong mutual repulsion with consequent band spreading. The migration of GAGs in formate–pyridine was essentially linear with time (Fig. 1b) or voltage (Fig. 1c), but that in barium acetate was clearly non-linear. All conditions which increased the distance between the two GAGs also promoted band spreading. Optimum conditions were selected as those producing the minimum separation needed to cut apart the strip into distinct bands for further analysis.

Anodal effect

The strong non-linearity seen with the barium acetate buffer could be attributed to an anodal effect; it did not appear to be a function of the parameters varied in Fig. 1, but was dependent upon the distance to the anode. The divergence from linearity was seen at between 4 and 5 cm under all conditions in Fig. 1. The non-linearity was even more striking when electrophoresis was started at the anode (Fig. 2). In this experiment samples were applied simultaneously to the anodal and cathodal ends and to the center of a strip. Those in sodium acetate behaved nearly the same regardless of where they were placed (Fig. 2a), whereas those in barium acetate all migrated toward

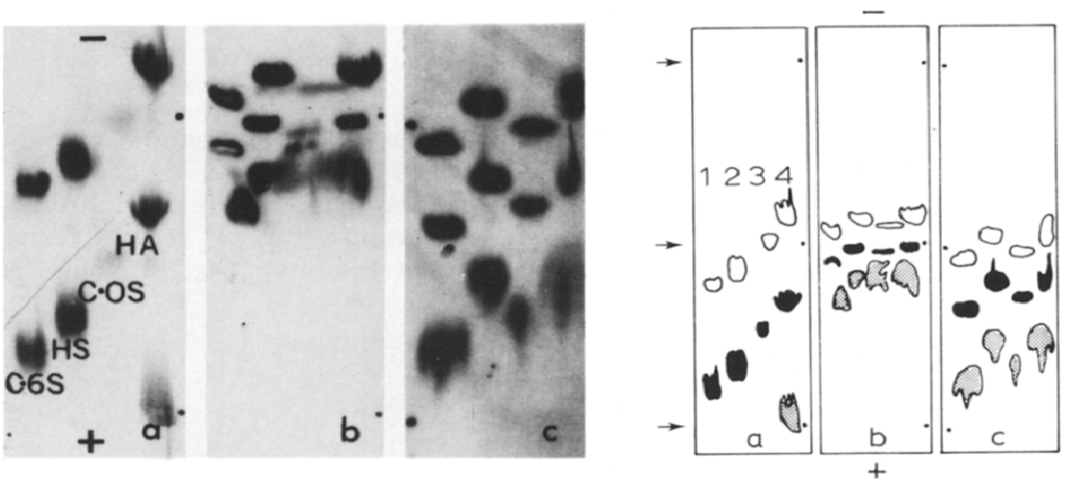

Fig. 2. The anodal effects with barium acetate buffer. Standard HA (4), C-0S (3), HS (2), and C-6S (1) (1 μg in 1 μl) were spotted at three origins along each strip; one was in the middle and the other two were 3 mm from the support bar at each end of the tray (10 cm between the support bars). Only the middle and anodal origins (ink dots) are shown here to save space. Because the buffer wetted the support bars by capillarity the end samples were effectively applied 3 mm from the cathode or anode, respectively. Buffers for hydrating the strips and at the two poles were (a) 0.1 *M* sodium acetate, (b) 0.1 *M* barium acetate, or (c) 0.05 *M* barium acetate. Electrophoresis was at 20 V/cm for 30 min (0.1 *M* buffers) or 40 min (0.05 *M* buffer). All three groups of four GAGs migrated toward the center of the strip (upper dot) in barium acetate (b and c). In sodium acetate (a) they all migrated toward the anode; those originating at the anode migrated off the strip. The same four GAGs (indicated in Fig. 2a) were used in the same order for all three strips. The line drawing is a reduced size tracing of the photographs, showing the entire strip for orientation. The samples spotted at each origin (arrows) are identified by shading: cathodal (outline), middle (solid), and anodal (stippled).

the center of the strip, the migration being more rapid in 0.1 *M* buffer (Fig. 2b) than in 0.05 *M* buffer (Fig. 2c). The anodal effect was only seen with divalent cationic buffers; with sodium acetate (Fig. 2a) or with formate—pyridine (Fig. 1b) migration was more nearly linear, and the samples simply moved off the end of the strip into the buffer.

The anodal effect was observed recently by Noordegraaf [14] when he spotted HA and C-6S at the anode in 0.1—0.3 *M* calcium acetate (pH 7.2 or 3.2). He interpreted the non-linearity of migration as an electrofocusing effect, but he did not provide evidence for the requisite stable ionic or pH gradient. The phenomenon may more simply be explained as electroendosmosis of the GAG—cation complexes, which no longer bear a negative charge.

A two-buffer system

Four common embryonic GAGs (HA, C-0S, HS, and C-6S, or C-4S), did not all separate cleanly in either formate—pyridine or barium acetate buffer alone; HS and C-6S or C-4S comigrated in the former (Fig. 3a, b), and C-0S and C-6S comigrated in the latter (Fig. 2 and Fig. 3c, d). In sodium acetate buffer (Fig. 2a) C-6S and HS showed an overlap that prevented quantitative separation, similar to their behavior in formate—pyridine buffer. However, with the sequential application of buffers, using conditions based on the behavior of GAGs in each buffer, all four GAGs could be separated on a single strip (Fig. 3 e—h). As long as the electrophoresis conditions were carefully

Fig. 3. Comparison of single and double buffers. Standard HA, C-0S, HS, and C-6S were applied either alone (1 μg in 1 μl) or as a mixture (0.5 μg of each in 2 μl) and electrophoresed at 20 V/cm using a 0.005 *M*—0.05 *M* buffer interface. The four single standards were applied to full-width (2.5 cm) strips, and the mixtures were applied to strips cut in half lengthwise. The position of the origin was beyond the top of these photographs and is omitted to save space. (a, b) 0.05 *M* Formate—pyridine buffer (pH 3.0) at 20 V/cm for 40 min. (c, d) 0.05 *M* Barium acetate buffer (pH 5.8) at 20 V/cm for 90 min. (e, f) Two buffers, with electrophoresis first in 0.05 *M* formate—pyridine buffer at 20 V/cm for 30 min. The strips were immersed in 95% ethanol for 5 min, 75% ethanol in 0.05 *M* barium acetate buffer for 5 min, and blotted. After a brief drying, they were blotted on filter papers dampened with 0.05 *M* barium acetate and immediately electrophoresed in this buffer for 20 min at 20 V/cm. (g, h) Two buffers, with electrophoresis first in 0.05 *M* barium acetate for 45 min and then in 0.05 *M* formate—pyridine for 30 min. The alcohol washes and blotting were as for Fig. 3e, f except that formate-pyridine buffer was used for the diluent with the 75% ethanol and for blotting.

controlled, nearly the same results could be obtained regardless of which buffer was used first. Electrophoresis using formate—pyridine as the first buffer was preferred, however, because the GAGs maintained their same relative positions during the second electrophoresis in barium acetate. If barium acetate was used first, HS had to catch up with and migrate through C-0S and HA in the formate—pyridine (compare Fig. 3a and c). This tended to produce band tailing (Fig. 3h).

DISCUSSION

We have shown that differences in the migration of GAGs in different buffers can be utilized on a single strip to attain a separation of closely related GAGs that is superior to that with most other one-dimensional techniques. The technique of Cappelletti et al. [9] may offer a better resolution of the more highly sulfated GAGs, but in our hands it offered no advantage for the four major GAGs of embryonic tissue tested here to offset the greater complexity in protocol. Theoretically, two-dimensional techniques should offer the best resolution, but for the GAGs so far tested this way (e.g., refs. 7, 8, 19) they offer no advantage over the present technique or that of Cappelletti et al. [9] and they do not have the advantage of a precise comparison of sample migration.

A problem that is inherent in the present two-buffer system, as well as in two-dimensional electrophoresis, is the requirement to switch buffers completely, but at the same time to prevent loss of sample [7]. We have attempted to solve the problem by precipitating the GAGs on the strip with alcohol, changing buffers in the presence of alcohol, evaporating some of the alcohol, and finally allowing fresh buffer to be absorbed from damp filter paper. When there was inadequate infiltration by the second buffer, the bands tended to diffuse to varying degrees, but this did not appear to interfere with the relative mobilities of the GAGs. We have experimented with transferring the strips directly, without blotting or any manipulation, to allow the buffers to change electrophoretically. This occasionally gave excellent separation, but the results were inconsistent because of uneven buffer replacement, which resulted in U-shaped bands. GAGs also were precipitated on the strips with 1% cetylpyridinium chloride and washed in varying concentrations of the second buffer, but we were unable to dissociate the complex reproducibly without loss of sample. This technique, however, could be of value when electrophoretically separating GAGs with widely differing precipitation characteristics or when separating GAGs from contaminants and, therefore, deserves further investigation. Another technique was to do the first run in a volatile buffer (e.g., formate—pyridine), allow the strips to air-dry, and then immerse them in the second buffer directly [19]. This procedure gave excellent band separation, but the staining intensity was significantly reduced and was not reduced the same for all GAGs, making this procedure of qualitative value only.

The anodal effect, first reported by Noordegraaf [14], needs further investigation. By selecting divalent cations with different binding affinities for either carboxyl or sulfate groups it may be possible to enhance the separation

of certain GAGs as they approach the anode. Often, electrophoresis of GAGs in barium acetate buffer is reported to be carried out for several hours [1, 5, 7, 16, 19], and it seems likely in these instances that an anodal effect must be acting to alter the relative migration of specific GAGs. For example, by cutting a strip lengthwise and staining half of it after about 45 min while continuing electrophoresis on the other half for 90 min we have observed that HA will continue migration toward the anode after C-6S has begun to migrate back toward the cathode.

We conclude that the discontinuous electrophoresis of GAG in one dimension can enhance resolution over standard one-dimension techniques employing a single buffer. In addition, the technique should be easily modifiable by appropriate selection of buffers to permit the separation of closely related proteins or other macromolecules in other electrophoresis systems.

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