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IDENTIFICATION OF ACIDIC MUCOPOLYSACCHARIDES BY AGAROSE GEL ELECTROPHORESIS

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SUMMARY

A micro-method for the identification of most acidic mucopolysaccharides by agarose gel electrophoresis with three different buffer systems is described. In barbital buffer the mucopolysaccharides are fractionated from each other as a function of their net charge, whereas in a diamine buffer the fractionation is probably achieved according to the degree to which they are bound to the diamine. A combination of barbital and diaminopropane buffers in two-dimensional electrophoresis for the identification of mucopolysaccharides is also described.

INTRODUCTION

Electrophoresis is now becoming one of the most widely used methods for the identification of acidic mucopolysaccharides (AMPS). This method takes advantage of the different net charges of the AMPS and is usually carried out combined with bivalent cations that change selectively the degree of migration of some AMPS. The cations commonly used are those of barium[1], zinc[2], calcium[3] and copper[4]. Cellulose acetate[1–5], agarose[6] and polyacrylamide[7,8] are the supports usually used for the electrophoresis.

It has recently been demonstrated that heparin and other AMPS could also be fractionated from each other by electrophocusing[9]. A detailed study of this phenomenon suggested that the fractionation was achieved as a result of specific binding of the AMPS to the amino groups of the different ampholines[10,11]. As the electrophocusing procedure is time consuming and expensive for routine analyses and because multiple band formation was observed with some AMPS, a search was made in order to determine whether simple amines could be substituted for the ampholines in the fractionation of these compounds.

This paper reports the fractionation of AMPS in diamine buffers in one- and two-dimensional agarose gel electrophoresis. A preliminary report on some of the findings will appear elsewhere[12].

MATERIALS AND METHODS

Mucopolysaccharides and other reagents

Hyaluronic acid and chondroitin sulfates A, B and C were purchased from Miles Laboratories (Elkhart, Ind., U.S.A.). Heparitin sulfates A, B, C and D were prepared as previously described[13]. Heparin was a kind gift from Upjohn (Kalamazoo, Mich., U.S.A.). Heparins were also obtained from Lederle, Abbott and Riker Laboratories. Hyaluronic acid, chondroitin sulfates, kerato sulfate and heparin were also kindly given by Dr. M. B. Mathews; they constitute the standards prepared by Drs. M. B. Mathews, J. A. Ciffonelli and L. Roden of the University of Chicago under a National Heart Institute Grant. 1,3-Diaminopropane and 1,10-diaminodecane were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). 5,5-Diethylbarbituric acid (barbital) was obtained from E. Merck (Darmstadt, G.F.R.) and agarose from L'Industrie Biologique Française (Gennevilliers, Seine, France).

Agarose gel electrophoresis of AMPS

Agarose gel slides (5.0 × 7.5 cm), 0.1 cm thick, were prepared with 0.9% agarose in 0.06 *M* barbital buffer (pH 8.6). The samples (3–5 μl) containing 1–10 μg of AMPS were applied in the agarose gel slide in slots of 0.3 cm at a distance of 1 cm from the edge of the slide. These slots were made with small pieces of Whatman 3 MM filter paper (0.3 × 1 cm), which were inserted into the gel such that they were perpendicular to its surface. The agarose slide was then subjected to electrophoresis at 5° in a chamber similar to that described by Wieme[14]. In some instances the barbital buffer was substituted by diamine acetate buffers in the preparation of the gel electrophoresis chamber and slides. These buffers (0.025–0.05 *M*) were prepared by adding acetic acid to diamino propane solution to give a pH of 8.5 and to diaminodecane solution (maintained at 60°) a pH of 10.3.

The electrophoresis was carried out at a potential of 150 V for about 1 h or until the dye indicator (cresol red) migrated 3.0 cm from the origin when barbital buffer was used or 4.5 cm when the diamine buffers were used. After electrophoresis, the gel was immersed in 0.1% solution of Cetavlon (cetyltrimethylammonium bromide, Merck) for 3 h. The gel was then covered with a Whatman 3 MM filter-paper strip that had previously been wetted with the Cetavlon solution and the slides were placed under a 250 W infrared lamp for 2 h in a current of air from a fan. They were then stained with a 0.1% solution of toluidine blue in acetic acid–ethanol–water (0.1:5:5). The slides were de-stained with the same solution without toluidine. Further details on the preparation of the chambers and slides are given elsewhere[6,15].

The two-dimensional agarose gel electrophoresis was performed as follows: the mixtures of AMPS were applied in the agarose gel prepared in barbital and subjected to electrophoresis. After the run, agarose gel strips (5 × 0.2 cm) containing the fractionated AMPS were cut off and placed in a slot of the same width and length situated transversely 2 cm from the edge of another agarose gel slide prepared with the diaminopropane buffer. The slide was then subjected to electrophoresis and stained as described above.

RESULTS

Results for the agarose gel electrophoresis of the AMPS in diaminopropane and barbital buffers are shown in Fig. 1. While chondroitin sulfates A and B and some heparins migrate to the same extent in barbital buffer, these compounds are completely separated from each other in diaminopropane buffer. Likewise, heparitin sulfates B and C are separated from each other in diaminopropane buffer but not in barbital. In contradistinction, kerato sulfate and hyaluronic acid are fractionated from each other and from the other AMPS in barbital buffer but not in diaminopropane (Fig. 1, Table I).

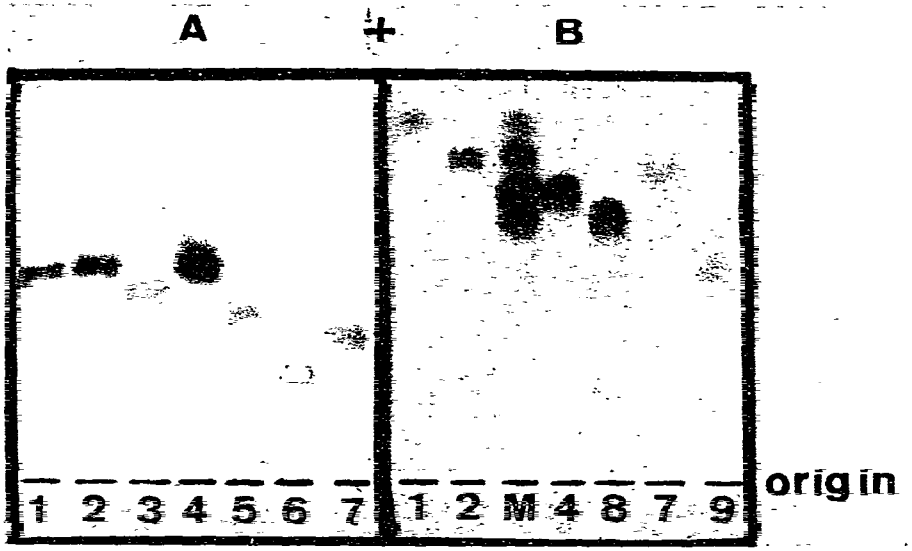

Fig. 1. Agarose gel electrophoresis of AMPS in barbital and diaminopropane buffers. About 2 μg of each AMPS in 3 μl of water were applied in the agarose gel prepared with 0.06 *M* barbital buffer (pH 8.6) (A) and 0.05 *M* diaminopropane acetate buffer (pH 8.5) (B) and subjected to electrophoresis for 1 h at 150 V. The AMPS were visualized as described under Materials and methods. 1 = Chondroitin sulfate A; 2 = chondroitin sulfate B; 3 = chondroitin sulfate C (Univ. of Chicago); 4 = heparin (Univ. of Chicago); 5 = heparitin sulfate; 6 = hyaluronic acid; 7 = keratosulfate; 8 = heparitin sulfate C; 9 = macromolecular heparin from rat skin provided by Dr. A. Horner, Univ. of Toronto. M = Mixture (2 μg each) of chondroitin sulfate A, chondroitin sulfate B, heparitin sulfate and heparin.

The relative electrophoretic migrations in three buffer systems of AMPS obtained from different sources are shown in Table I. Except for chondroitin sulfate C and heparin, all the AMPSs migrate to the same extent regardless of their source. When barbital buffer is used, the heparins obtained from different sources contain two components except that obtained from Upjohn, which contains only one (Table II). In diaminopropane, all the heparins show the presence of only one component with the same electrophoretic migration except that obtained from the University of Chicago, which has a lower electrophoretic mobility. In diaminodecane, all of them remain at the point of application on the agarose slide. The other AMPS have about the same relative electrophoretic mobilities as in diaminopropane buffer.

TABLE I

RELATIVE ELECTROPHORETIC MIGRATION OF AMPS IN THREE DIFFERENT BUFFERS

*AMPS**	*Relative electrophoretic migration (R_{ChSA})***		
	Barbital	*1,3-Diaminopropane*	*1,10-Diaminodecane*
Chondroitin sulfate A(1)	1.00	1.00	1.00
Chondroitin sulfate A(2)	1.00	1.00	1.00
Chondroitin sulfate B(1)	1.00	0.89	0.91
Chondroitin sulfate B(2)	1.00	0.89	0.91
Chondroitin sulfate C(1)	0.87	0.97	0.96
Chondroitin sulfate C(2)	1.00	0.97	0.96
Heparitin sulfate(1)	0.77	0.82	0.81
Heparitin sulfate B(3)	0.77	0.82	0.81
Heparin(1)	1.00, 1.15	0.67	0
Heparin(4)	1.15	0.76	0
Hyaluronic acid(1)	0.51	0.83	—
Hyaluronic acid(2)	0.52	0.83	—
Kerato sulfate(1)	0.69	0.89	0

* AMPS preparation: (1) University of Chicago; (2) Miles Lab.; (3) prepared as described in ref. 5; (4) Upjohn Co.

** R_{ChSA} = migration of the AMPS/migration of chondroitin sulfate A.

TABLE II

RELATIVE ELECTROPHORETIC MIGRATION OF HEPARINS AND HEPARITIN SULFATES

Heparin source	*Relative electrophoretic migration (R_{ChSA})*		
	Barbital	*1,3-Diaminopropane*	*1,10-Diaminodecane*
Upjohn	1.15	0.76	0
Riker Lab.	1.00, 1.15	0.76	0
Abbott Lab.	1.00, 1.15	0.76	0
University of Chicago	1.00, 1.15	0.67	0
Heparitin sulfate A	0.77	0.82	0.78
Heparitin sulfate B	0.83	0.82	0.78
Heparitin sulfate C	0.85	0.53	0.67
Heparitin sulfate D	1.15	0.78	0.92
Chondroitin sulfate A	1.00	1.00	1.00

Table II also shows the relative migrations of the heparitin sulfates prepared from beef-lung tissue. Heparitin sulfates B and D are separated from each other in barbital buffer and heparitin sulfates B and C in diaminopropane buffer.

The relative electrophoretic migrations of the AMPS shown in Table I indicate that most of them can be fractionated from each other by a combination of the barbital and diaminopropane systems. Fig. 2 shows the result of a two-dimensional agarose gel electrophoresis using the two buffer systems. All the AMPS added in a mixture were separated from each other by this procedure.

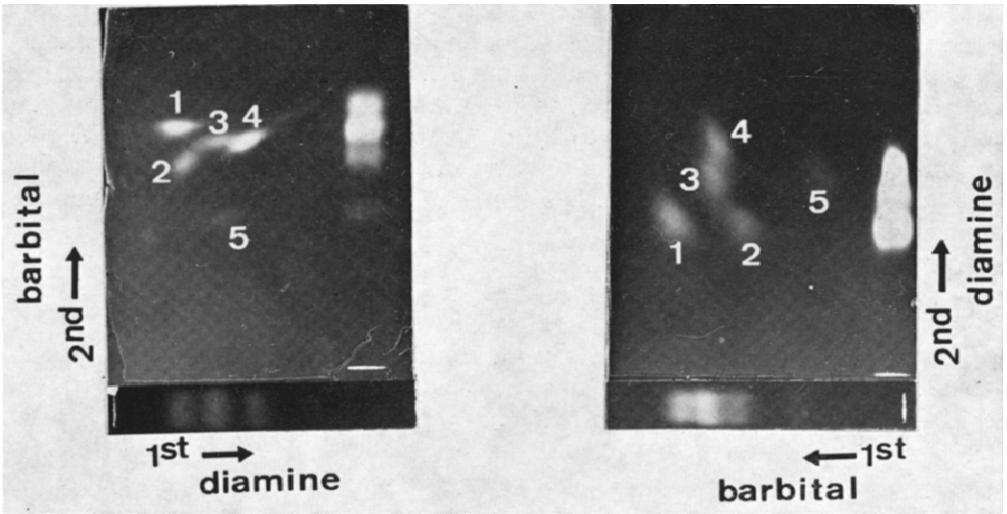

Fig. 2. Two-dimensional agarose gel electrophoresis of AMPS in barbital and diaminopropane. The mixture contained about 2 μg of heparin (1), heparitin sulfate (2), chondroitin sulfate B (3), chondroitin sulfate A (4) and hyaluronic acid (5).

DISCUSSION

The comparison of relative electrophoretic migrations of the AMPS in barbital and diaminopropane buffers suggests that at least two parameters are involved in the fractionation of these compounds. In barbital, it seems that only the charge plays a significant role in the fractionation. Thus, heparin, the most polyanionic of these compounds, has the highest electrophoretic migration and hyaluronic acid and kerato sulfate the lowest. In diaminopropane, the extent of fractionation seems to be related to the degree to which the AMPS are bound to the diamine. Thus, except for hyaluronic acid and kerato sulfate, the mobilities of all the AMPS are retarded by diaminopropane. The degree of retardation nevertheless varies with each AMPS. The mobility of heparin is the most retarded by the diamine as it has the highest electrophoretic migration in barbital and among the lowest in diaminopropane buffer. The retardation does not seem to be entirely related to the availability of the charges of the AMPS because chondroitin sulfates A and B, which have the same net charge, migrate to different extents in diaminopropane buffer, the latter being more retarded in this system. Similar electrophoretic behavior was observed by Hata and Nagai[4] when bivalent cations were used instead of phosphate buffer. For instance in copper (II) acetate buffer, heparin was the AMPS with the lowest electrophoretic migration. Nevertheless, a broad spot was obtained for heparin in this buffer system.

The differences in electrophoretic migrations of heparins, as well as of chondroitin sulfate C, from different sources call attention to the fact that these AMPS might vary according to the tissue of origin. Appropriate standards should then be used for the identification of these compounds by the electrophoretic method.

The agarose gel support has a number of advantages over cellulose acetate: it can be used for preparative purposes simply by scaling up the load of AMPS and the

thickness of the agarose blocks[16]; it accepts heavily contaminated mixtures without a change in the pattern of fractionation[12]. Also, an added advantage, owing to its load capacity, is that small amounts of individual AMPS can be identified when in a mixture. For instance, in agarose gel, urinary AMPS shows the presence of four components one of which amounting to 2% of the total[17]. The same urinary AMPS on cellulose acetate shows the presence of only two main components[18]. Compared with cellulose acetate the present method has the disadvantage that it requires a longer development time.

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