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THE EFFECT OF pH AND IONIC STRENGTH ON THE ELECTROPHORETIC SEPARATION OF ACIDIC GLYCOSAMINOGLYCANS

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SUMMARY

Using the electrophoretical methods applied to this study it is possible to determinate the dissociation constants (pK) of acid glycosaminoglycans containing a carboxylic group. The pK-values of the six acid glycosaminoglycans separated from animal connective tissues determined in this work were: hyaluronic acid (HA), pK = 3.0; chondroitin sulfate A (CS-A), pK = 2.8; chondroitin sulfate C (CS-C), pK = 3.3; dermatan sulfate (CS-B), pK = 3.3; heparatin sulfate (HeS), pK = 3.1 and heparin (HeP), pK = 2.4 and were measured at a constant ionic strength of I = 0.164 (NaCl) and at 10 ± 2°C.

Variation of ionic strength showed that physiological conditions seem to be most suitable for the electrophoretic separation of the glycosaminoglycans studied. A decrease of ionic strength causes increasing mobility but less accurate spots. In the case of increasing ionic strength the results are vice versa.

The second spot for HA very often appeared when pH values higher than 2 were used for electrophoresis. The spot had the same form as the original, high intensity, but an undecided migration in the pH range near the pK value of HA (3.0).

INTRODUCTION

Both one and two dimensional electrophoresis are widely used for the fractionation of acid glycosaminoglycans (GAG) [1—7]. To increase the fractionation effect the pH is often adjusted to a suitable range. Metal salt solutions are also used as buffer substances in the electrolytic chamber [6]. Other methods, such as column chromatography [7—9], thin-layer chromatography [10, 11] and gas chromatography [12, 13], also exhibit similarities in the fractionation of GAGs.

In the electrophoretic procedure the carrier solution is reduced in the anode and oxidized in the cathode chamber. Thus, to keep the pH constant, the electrolytic solution should have a good buffer capacity. Metal salt solutions such as $Cu(CH_3COO)_2$; $Ba(CH_3COO)_2$; $Zn(CH_3COO)_2$; $La(NO_3)_3$ and $Ca(CH_3COO)_2$ [6, 14] used in electrophoretical procedures have a very low

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buffer capacity and a pH change in the chambers cannot be avoided. Strong acids and some other good buffer substances are able to keep the pH in both chambers very stable.

The purpose of the present investigation was to study the migration speed of GAGs in an electric field, as a function of pH. The aim was also to test the influence of the ionic strength on the mobility, in both acidic and alkaline conditions, of GAGs. In some earlier studies these problems have been approached, but there are still many open questions [1, 15].

EXPERIMENTAL

The electrophoresis of the GAGs was performed using a cellulose acetate membrane (Beckman cellulose acetate strips) and Beckman Microzone R-200 equipment with a specially constructed water cooling system. The temperature was adjusted to 10 ± 2°C. A Microzone Duostat power supply model RD-2 was used as the power unit. The pH-measurements were carried out with a Radiometer pHM-26, and with a Beckman glass electrode type No. 41263. An immersion-type calomel electrode was used as a reference. A standard pH solution containing 0.01 *N* HCl in physiological NaCl was recommended. The pH of this solution was calculated to be 2.11 at 10°C [16]. The ionic strength was adjusted to $I \approx 0.164$ with NaCl (except when the effect of ionic strength was studied). The pH of the electrophoretic solution was checked both before and after the run from both the anode and cathode chamber.

The electrophoresis was performed using a 30-min migration time, 110-V power and 50-mA current. After electrophoresis the strips were stained with a 1% solution of Alcian blue (G.T. Gurr's) in 25% acetic acid, then rinsed with tap water and 10% acetic acid.

On the very alkaline side from pH 10 to 11.4 electrophoresis procedures were performed under a nitrogen gas stream in order to avoid contact with carbon dioxide.

Reagents

The reagents chosen for buffering the electrophoretic solutions were as similar as possible. They were the following: urocanic acid and histidine (Sigma, St. Louis, MO, U.S.A.); citric acid imidazole and barbituric acid buffers (E. Merck, Darmstadt, G.F.R.) and glycine (Eastman Organic Chemicals, Rochester, NY, U.S.A.). All buffers were adjusted to the desired pH range using 0.1 *N* HCl or 0.1 *N* NaOH solutions purchased from Merck.

Reference glycosaminoglycans, hyaluronic acid (HA); chondroitin sulfate A (CS-A); chondroitin sulfate C (CS-C); dermatan sulfate (CS-B); keratan sulfate-1 (KS-1); heparitin sulfate (HeS) and heparin (HeP), were gifts of Drs. M.B. Mathews, J.A. Cifonelli and L. Roden (Department of Pediatrics, The University of Chicago, Chicago, U.S.A.).

TABLE I

THE MIGRATION SPEED OF THE GAGs STUDIED IN THE ELECTRIC FIELD WITH VARIABLE pH VALUES FOR THE BUFFER USED

The desired pH range was produced by titrating the buffer with 0.1 *N* HCl or 0.1 *N* NaOH. Ionic strength was adjusted in each case, except for barbituric acid buffer, to $I = 0.164$ with NaCl. The initial pH and the pH after the run both in the cathode and anode were recorded.

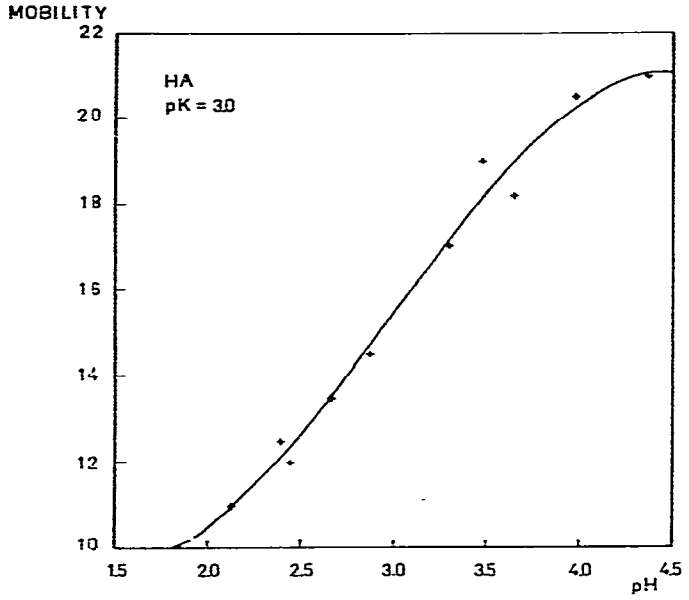
Buffer substance	Mobility (mm)							pH		
	HA	CS-A	CS-C	CS-B	KS-1	HeS	HeP	start	anode	cathode
0.1 *N* HCl	19.0	22.5	22.5	23.2	23.7	21.7	25.7	1.42	1.42	1.42
0.08 *N* HCl	18.0	21.0	21.0	21.0	22.0	20.0	25.0	1.44	1.44	1.44
0.06 *N* HCl	17.0	22.0	22.0	23.0	23.0	21.0	26.0	1.50	1.50	1.50
0.04 *N* HCl	13.5	21.2	21.5	22.7	23.7	20.5	28.0	1.61	1.61	1.61
0.02 *N* HCl	10.0	19.0	19.0	20.0	21.0	17.0	26.0	1.85	1.85	1.86
0.01 *N* HCl	11.0	21.5	22.5	22.7	24.0	19.0	29.5	2.12	2.11	2.12
0.02 *M* Histidine HCl	12.5	21.5	22.2	22.2	23.0	19.5	27.0	2.39	2.35	2.46
0.02 *M* Citric acid	12.0	22.0	22.7	22.7	23.0	19.5	28.7	2.44	2.44	2.50
0.02 *M* Histidine HCl	13.5	23.0	24.0	23.0	23.0	19.5	26.5	2.66	2.63	2.79
0.01 *M* Urocanic acid	14.5	23.5	23.5	23.5	22.5	20.5	28.0	2.87	2.74	3.04
0.02 *M* Citric acid	17.0	26.0	26.0	25.0	24.0	23.0	29.0	3.30	3.25	3.32
0.01 *M* Urocanic acid	19.0	28.5	28.5	27.0	25.2	25.2	31.5	3.48	3.37	3.70
0.02 *M* Citric acid	18.2	28.0	28.2	27.0	24.7	25.0	32.0	3.65	3.62	3.72
0.01 *M* Urocanic acid	20.5	28.5	29.5	28.5	24.0	25.0	30.7	3.98	3.79	4.17
0.01 *M* Urocanic acid	21.0	30.0	30.5	30.7	25.2	26.5	32.5	4.37	4.25	4.67
0.02 *M* Citric acid	20.5	28.0	28.5	29.0	23.5	25.2	30.5	4.83	4.79	4.90
0.02 *M* Citric acid	19.5	26.5	27.0	27.0	22.0	24.0	29.5	5.08	5.04	5.19
0.01 *M* Urocanic acid	21.0	28.7	30.0	30.2	24.0	25.7	30.5	5.48	5.35	5.61
0.01 *M* Urocanic acid	19.5	29.0	29.5	29.7	24.0	25.5	31.0	5.83	5.66	6.02
0.01 *M* Urocanic acid	21.0	29.0	30.5	30.7	25.5	26.5	31.5	6.20	6.07	6.36
0.02 *M* Imidazole	20.5	28.5	29.5	29.5	24.2	26.2	31.0	6.63	6.60	6.77
0.02 *M* Imidazole	21.0	30.2	31.0	31.0	25.5	27.0	32.0	7.10	7.06	7.12
0.02 *M* Imidazole	21.5	31.0	31.7	31.7	25.5	27.5	33.0	7.59	7.57	7.69
Barbituric acid buffer	20.0	31.5	31.5	31.5	23.5	26.5	32.5	8.50	8.36	8.51
0.02 *M* Histidine HCl	22.0	31.0	31.5	32.0	25.0	27.0	32.0	8.64	8.57	8.69
0.02 *M* Histidine HCl	22.5	31.0	31.5	31.5	25.5	26.5	32.5	9.08	9.07	9.26
0.02 *M* Histidine HCl	22.0	30.5	31.0	31.5	25.0	26.7	32.0	9.60	9.54	9.61
0.02 *M* Glycine	21.5	29.7	30.5	30.7	25.0	26.0	30.5	10.08	9.99	10.18
0.02 *M* Glycine	21.0	30.5	31.0	32.0	26.0	27.0	32.5	10.68	10.57	10.70
0.02 *M* Histidine	20.0	29.0	29.5	30.0	24.0	26.0	31.0	11.27	11.15	11.32
0.02 *M* Glycine	18.0	25.5	26.0	26.0	22.0	22.5	27.0	11.41	11.35	11.53

TABLE II

p*K* VALUES OF THE CARBOXYLIC GROUP OF ACIDIC GLYCOSAMINOGLYCANS

The values are based on electrophoretical measurements. Conditions: temperature, 10 ± 2°C; $I = 0.164$.

Substance	Abbreviation	p*K* (-COOH)
Hyaluronic acid	HA	3.0
Chondroitin sulfate A	CS-A	2.8
Chondroitin sulfate C	CS-C	3.3
Dermatan sulfate	CS-B	3.3
Keratan sulfate	KS-1	—
Heparitin sulfate	HeS	3.1
Heparin	HeP	2.4


MOBILITY
22
20
18
16
14
12
10
HA
pK = 3.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
pH

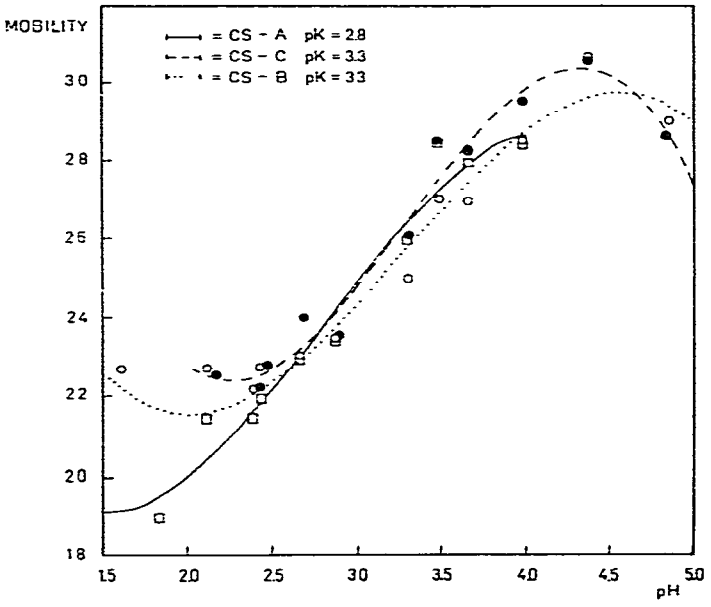

MOBILITY
= CS - A pK = 2.8
= CS - C pK = 3.3
= CS - B pK = 3.3
30
28
26
24
22
20
18
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
pH

Fig. 1.

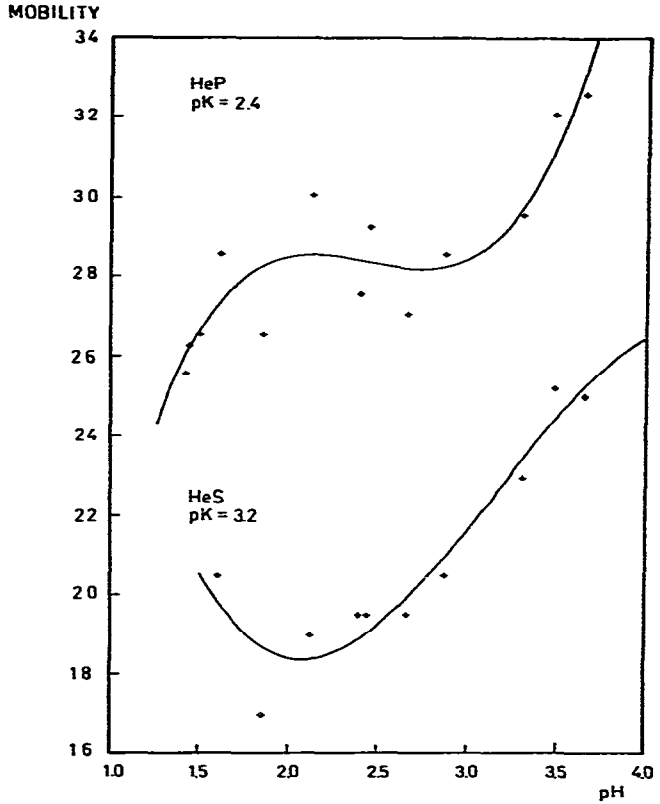


Fig. 1. The mobility of glycosaminoglycan in the electric field as a function of pH in the dissociation area of the carboxylic group. (a) Hyaluronic acid (HA); (b) chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C) and dermatan sulfate (CS-B) and (c) heparitin sulfate (HeS) and heparin (HeP).

RESULTS

As a function of pH, the mobility rates indicate that all significant variations occurred below the pH range of pH 4.5 (Table I and Fig. 1). Keratan sulfate is, however, an exception. The pH value does not seem to have any influence on the mobility of this particular glycosaminoglycan.

The rapid rise between pH 1.6 and 4.4 indicates the ionisation of the acidic carboxylic groups in the curve. The turning points of these pH jumps were calculated by means of the least square method as the points were pH = pK, and are listed in Table II.

In the dissociation area of the carboxylic group of HA especially, a special form of the HA spot appeared in which the easily identifiable shadow spot had very often migrated in the opposite direction from starting point. In Fig. 2a there is a good example of this effect. Fig. 2b shows the reference migrations in the dissociation area where the special form of HA is also seen. Examples of this kind of cleavage of HA also exist when histidine buffer, pH 5.70, and even barbituric acid buffer, pH 8.50, were used.

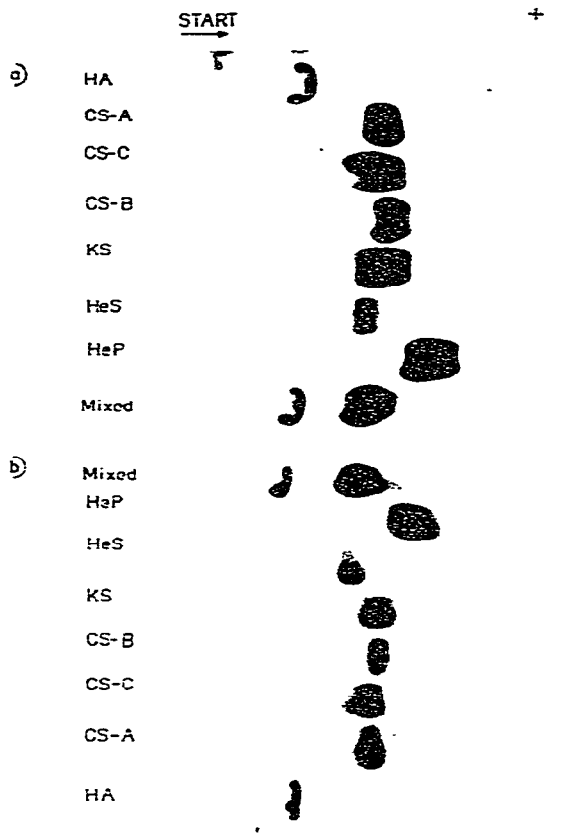


Fig. 2. (a) Electropherogram obtained using 0.02 *M* citric acid (pH 2.7), I = 0.164 (NaCl) as buffer solution. Note the cleavage of HA into the two spots having a typical form; (b) the reference run for GAGs on the acidic side. The buffer solution used in this experiment was 0.002 *N* HCl + 0.162 *M* NaCl, pH ≈ 2.50. The special form of HA is still present.

The influence of ionic strength on the mobility of the GAGs was investigated in both acidic and alkaline conditions. The effect of ionic strength seemed to be almost independent of that of pH. The ionic strength has, however, a great influence on the resolution ans sharpness of the spot. When a buffer solution with high ionic strength was used the migration was slow but the resolution was good (Fig. 3a). With a buffer with low ionic strength the migration improved, but on the other hand the sharpness of the spots was impaired as compared to the pherograms obtained from buffers with higher ionic strength (Fig. 3c).

DISCUSSION

The influence of pH variation on the separation of acidic GAGs has been investigated before [1] but never using such a wide pH range. The results of the present work show that the determination area of the pK values of the

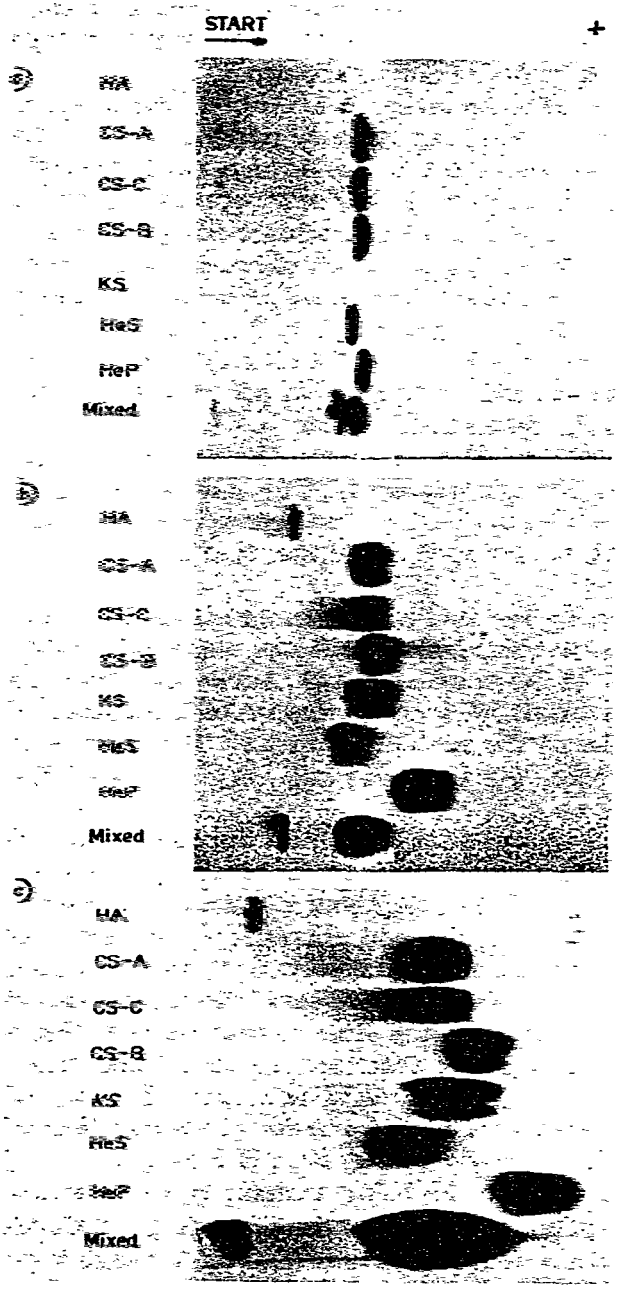


Fig. 3. The effect of ionic strength on the separation of GAGs on the acidic side (pH 2.1). (a) Buffer solution 0.01 *N* HCl + 0.5 *M* NaCl (high ionic strength); (b) 0.01 *N* HCl + 0.154 *M* NaCl (normally used ionic strength) and (c) 0.01 *N* HCl + 0.01 *M* NaCl (low ionic strength).

sulfate group of glycosaminoglycans, as seen in the case of keratan sulfate, is so acidic that it would be impossible to determine the pK values because of the drying of the cellulose acetate membrane during the run.

The effect caused by the variation of ionic strength is quite obvious. More ions in the electrolytical system require stronger current, and vice versa. The accuracy of the spot seems to be much better in the case of high ionic strength, indicating the pooling effect generally applied in column chromatography.

Although the pK values determined in this work are inaccurate, especially the pK value of HeP, it is seen that the acidity of GAGs decrease in the order HeP < CS-A < HA < HeS < CS-B < CS-C. Using the tritation method Laurent [17] obtained the value of pK = 2.95 for HA when I = 0.2 (NaCl). This is in good agreement with the value (pK = 3.0, I = 0.164 (NaCl), temperature, 10 ± 2°C) obtained by our electrophoretical method. The pK values of other GAGs have not, to our knowledge, been determined before.

The rapid rise of mobility as a function of pH seems to be connected with the GAGs having the carboxyl group in the molecule. The rise seems to be quite linear in the two co-adjacent pH units (i.e. 1 pH unit after the pK value). This experimental result also corresponds well with the findings obtained by Kiso and co-workers [15, 18].

From Fig. 2 and Table I it is easy to see that on the most acidic side many GAGs, HA, HeS and to some extent chondroitin sulfates, especially CS-C, have increasing mobility as a function of decreasing pH. According to Jokl's [19] observations this should be a result of (a) an increase in the charge of the GAG molecule or (b) a decrease in the molecular weight. The latter suggestion is out of the question, because the spot was still very accurate after the run.

The cleavage of the HA into two compounds having an opposite charge is difficult to explain. The splitting effect was found to be clearest in the dissociation range of the carboxylic group of HA, i.e. from pH 2 to 4, where the spot also has a special form caused by undecided migration direction (see Fig. 2a). Examples of cleavages having a wide distance with histidine buffer, pH 5.7, and even with barbituric acid buffer, pH 8.5, also existed, indicating that the cleavage is not only connected with the dissociation of HA. Some electrolytic solutions seem to have a stronger tendency to cause this cleavage of HA than others. A good example is citric acid near the pK range of HA. This pH area is also recommended as a useful one for the separation of acidic GAGs based on pH variation (Fig. 1 and e.g. Fig. 2b, pH 2.50).

The role of ionic strength is seen in Fig. 3. Using variable salt concentration it seems to be possible to regulate the separation procedure of GAGs as a function of mobility and sharpness of the spot. Because of the high ionic strength on the highly acidic side the KS-1 spot disappeared.

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