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Note

Simple electrophoresis of glycosaminoglycuronans and the distinction of lung heparins from mucosal heparins

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So many electrophoretic systems have been devised and published for the analytical separation of glycosaminoglycans (GAGs) that it might seem pointless to suggest the use of yet another. It probably has to be accepted that no one simple continuous system will separate all samples of a single GAG class (e.g. dermatan sulphate) from all samples of every other class, but a system which will assuredly operate effectively in laboratories other than that of the devisor has definite advantages over a complex one which, for successful application, demands manipulative subtleties which may not be adequately described — perhaps because they are not even appreciated — in the published account.

The present system has been used only for the sulphated glycosaminoglycuronans; the migration of hyaluronate and of keratans has not been investigated. If we consider electrophoresis of GAGs on the most convenient medium, cellulose acetate membranes, two main kinds of simple continuous buffer system are in general use: one of low pH where molecular charge is the dominant factor determining separation, and another of more neutral pH containing a cation which binds with varying specificity to different classes of GAG. The first kind, originated by Mathews [1], separates heparin (Hep) from the less sulphated classes, but makes little distinction between the chondroitin sulphates (ChS), dermatan sulphate (DeS) and similarly sulphated heparans (HeS). The second kind has a wider application; in the form of barium acetate it was introduced by Wessler [2], and among the accessible metal cations barium remains the most useful for electrophoretic GAG separations. Differences in the solubility of the barium—GAG complexes have also been used to enhance certain separations [3]. Dietrich and Dietrich [4] in-

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troduced α,ω-diaminoalkane buffers, and the three- and four-carbon members of this series show a remarkable resemblance to barium in their separative, and presumably complexing, ability. These buffers yield more compact spots on cellulose acetate than the low pH systems, and will usually separate Hep, DeS and ChS from each other, but since the spread of R_F values is rather limited there is little discrimination within a class. The system described here gives separations which are essentially similar to those obtained with barium acetate or propane-1,3-diamine acetate, but with a greater spread of R_F values, and since the spots are at least as compact (depending of course on sample homogeneity) greater discrimination is possible.

Heparin is commonly obtained commercially either from porcine intestinal mucosa or from bovine lung tissue. The two types differ [5], and for pharmacological or other reasons it may be desirable to distinguish the one from the other [6]. In the electrophoretic system described here the two types differ in behaviour to a degree sufficient for this purpose, but the distinction may be made more positive by using the discontinuous system of Hopwood and Harrison [7].

EXPERIMENTAL

Bis(3-aminopropyl)amine (dipropylenetriamine, DPTA)* was obtained from Fluka and from Aldrich; Ch4S I, Ch6S I, DeS I and HeS I were standard samples (batch 1977) kindly donated by Professors Cifonelli and Mathews, University of Chicago. Ch6S II (ex shark) was obtained from Koch-Light, and DeS II (ex pig skin) from Seikagaku. HeS II and III were prepared in this laboratory from residues of commercial mucosal heparin production (galactosamine <1% total hexosamine, SO_3^-/CO_2^- ratios 1.0 and 1.65, respectively), as was DeS III. Ch4S II (some 6-isomer by infrared) and DeS IV (some ChS as impurity) were prepared in this laboratory from bovine lung heparin production residues. The sources of the heparin samples are given in the captions to the figures.

The brands of cellulose acetate used were: Shandon Celagram (identical to Elvi Microphor), Gelman Sepraphore III, Schleicher and Schüll CA-Elektrophoresefolien (very similar to the Sepraphore), and the wet-stored gels Whatman Cellogel and Cellogel RS. In the electrophoresis apparatus (Shandon Minimicroband, no longer available) the cellulose acetate strips, ca. 8 cm long, were suspended in air between filter paper wicks over gap of about 6.5 cm without intermediate support. There were no special features except that the buffer chambers were partitioned by antidiffusion barriers into electrode and wick compartments; the buffer in the latter therefore remains relatively unaffected by electrolytic changes.

The buffer used was 0.025 *M* in DPTA, 0.03 *M* in Mg^{2+} and ca. 0.12 *M* in $CH_3CO_2^-$, pH ca. 7.0. A concentrated stock solution (×10) became yellow on standing at room temperature, and then gave reduced resolution. For best results the buffer should be fresh. For the Shandon apparatus, to 150 ml water was added successively 10 ml 0.75 *M* magnesium acetate solution, 0.96 ml

*Alternative catalogue names: 3.3′-iminobispropylamine, 3.3′-diaminodipropylamine.

glacial acetic acid, 0.89 ml amine, and the whole made up to 250 ml.

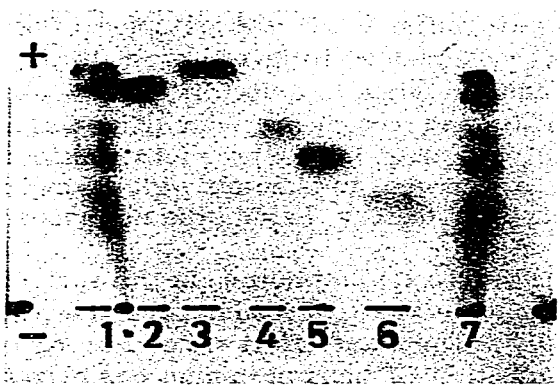
All operations were conducted at room temperature. Cellulose acetate strips of suitable width were equilibrated by floating on buffer for a minimum of 3 min (longer for wet-stored material), then laid on paper tissue or filter paper and loaded with about 0.2—0.4 μl GAG solution (2—5 mg/ml in water) in a zone of ca. 1 × 4 mm. Electrophoresis was performed with a current of about 1 mA/cm width; about 120 V was required. After about 1 h the fastest-moving GAG, Ch6S, had migrated about 30 mm, depending on temperature, and further electrophoresis was not usually advantageous. A number of runs may be made, reversing polarity each time to reduce changes in buffer composition, before the buffer needs to be renewed. Staining and destaining were carried out in the usual way, using Alcian Blue and 5% aqueous acetic acid.

For discrimination between lung and mucosal heparins the following electrophoretic system was used. The tank buffer was 1.0 *M* barium acetate, and the cellulose acetate was equilibrated before loading with 0.1 *M* barium acetate. After 30 min electrophoresis at about 1 mA/cm width the gel was removed and immersed for 2 min in 0.1 *M* barium acetate containing 30% ethanol, and, after blotting to remove excess buffer, electrophoresis was continued with the original tank buffer for a further 30 min. All other conditions were as described above.

RESULTS AND DISCUSSION

Fig. 1 displays the separation which may be obtained with a range of GAGs, and Fig. 2 indicates that DeS samples may show slight variations in mobility; substitution of DeS I for DeS III in Fig. 1 would destroy the separation between DeS and HeS I. One may speculate whether, in view of the close structural relationship between DeS and Ch4S, the faster migrating sample may have a higher ratio of glucuronate to iduronate.

Fig. 3 compares samples of ChS and also of the HeS—Hep sequence. The



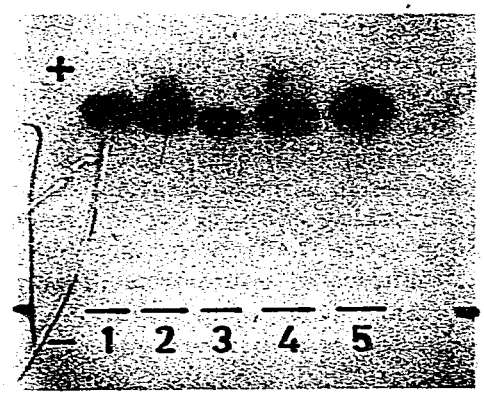


Fig. 1. DPTA + Mg^{2+} acetate buffer, as in text. Samples 1 and 7 = mixture of 2—6 with bovine lung heparin added, 2 = Ch4S I, 3 = Ch6S I, 4 = DeS III, 5 = HeS II, 6 = HeS III.

Fig. 2. Conditions as Fig. 1. Samples 1 and 5 = DeS I, 2 = DeS II, 3 = DeS III, 4 = DeS IV.

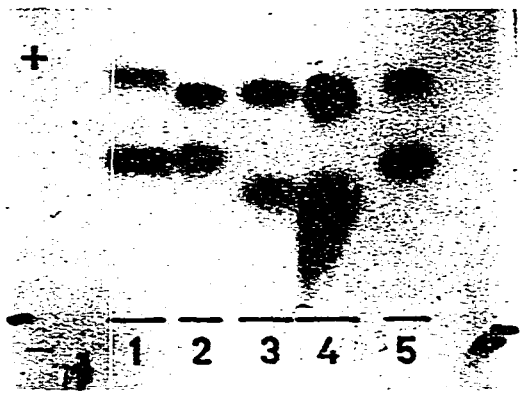


Fig. 3. Conditions as Fig. 1. Samples 1 and 5 = Ch6S I + DeS I, 2 = Ch4S I + HeS II, 3 = Ch4S II + HeS III, 4 = Ch6S II + porcine mucosal heparin.

clear-cut distinction between Ch4S I and Ch6S I (cf. Fig. 1) is not maintained for the other samples of this class. For heparan sulphates the migration rate is inversely related to the degree of sulphation, but (from results with other samples not included here) in a rather discontinuous manner. HeS I/II and HeS III may be representatives of two sub-classes of HeS, within each of which there is some variation in degree of sulphation — as is indeed the case with heparins.

The inherent heterogeneity within GAG classes, with consequent irregularities in electrophoretic behaviour, has recently been emphasised by Schuchman and Desnick [8]. Their conclusion however, that "only highly purified GAG preparations should be used as standards", appears to be wrong; it is surely desirable to prepare or otherwise find standards as close as possible in behaviour (implying also composition) to the corresponding constituents of the samples to be examined. Difficulties were encountered in trying to employ the electrophoretic procedure of Schuchman and Desnick [8], since EDTA salts available here did not yield the separations which they describe.

The composition of our electrophoresis buffer is not the outcome of an extensive research programme, but for those inclined to investigate it further a few comments may be useful. The amine acetate is quite difficult to use alone, for reasons involving conductivity and complexing strength. Quite a number of other metal acetates have been tried as likely co-ions; calcium behaves in almost identical manner to magnesium, but metals with stronger complexing ability were on the whole less satisfactory. Barium did not appear to improve the HeS and DeS separation.

Samples of commercial heparin subjected to electrophoresis in this system normally leave some material immobile at the point of loading, and the rest migrates as a diffuse zone (Figs. 1 and 3). The exact effect varies with temperature and obviously involves the formation of heparin—amine complexes with low solubility (cf. ref. 3), but with porcine mucosal heparin samples the mobile material migrates consistently faster than with bovine lung samples. However, although the DPTA buffer may be used to provide an indication of the tissue of origin of an unknown heparin — assuming it to be one of the two

usual kinds, porcine intestinal mucosal and bovine lung, and using samples of these for comparison — we have found no simple continuous system which is altogether satisfactory. Some trials with the discontinuous system of Cappelletti et al. [9] as modified by Hopwood and Harrison [7] were encouraging, provided that they were run with the gel strip suspended in air at ambient temperature as in our DPTA procedure; when we adapted our equipment to conform with the detailed Hopwood—Harrison procedure, confining the gel strip between melamine film (on a metal plate at 15°C) and parafilm under a flat 1000-g weight, we were not able to obtain satisfactory results. The procedure was further simplified by using only one intermediate buffer bath.

Fig. 4 was obtained at an ambient temperature of 27°C; at lower temperatures there is more non-mobile material with all samples, but the distinction is maintained. Samples 4 and 6 are typical of mucosal heparins, whereas sample 2 is the least sulphated of over 30 commercial heparin samples we have examined. All samples were essentially free of other GAGs. Discrimination between mucosal and lung heparins is also obtained with the procedure of Oreste and Torri [3], although we have found it difficult to obtain the distinctive and reproducible "fingerprints" described by these authors.

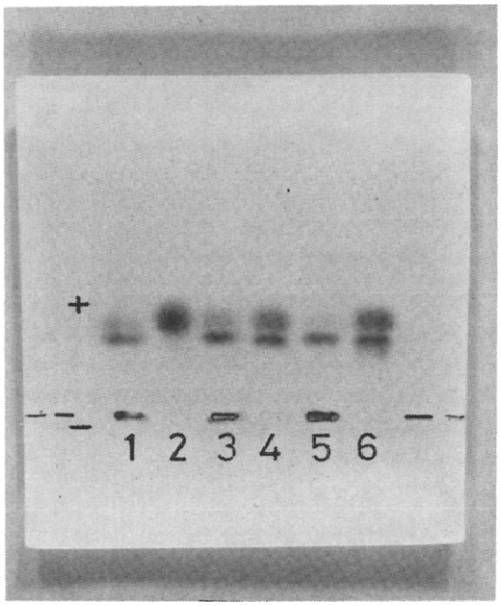


Fig. 4. Barium acetate buffers; conditions modified from Hopwood and Harrison [7] as described in text. Samples 1, 3 and 5 are bovine lung heparins; 1 = isolated in this laboratory, 3 = Upjohn lot 730EH, 5 = Choay lot FF783. Samples 2, 4 and 6 are porcine mucosal heparins; 2 = Terhormon lot 018, 4 = Bioiberica lot F4, 6 = Diosynth lot Hb 1659-I.

In the course of the trials of the Hopwood—Harrison electrophoresis procedure [7] it became clear from relative staining intensities that sample losses occur, particularly of the less sulphated GAGs, when the partly-run gels are soaked in the aqueous—ethanolic barium acetate solutions. Their procedure was of course designed primarily as an aid to the diagnosis of the mucopoly-

saccharidoses, and while for this purpose a reasonable consistency in the electrophoretic band patterns is essential, there is no requirement for an accurate representation of the proportions of the various GAGs present. Since our own system may not be effective in separating DeS from low-sulphated heparans such as are excreted in mucopolysaccharidoses, it may not be suitable for diagnosis except in conjunction either with chondroitinase B treatment or with galactosamine–glucosamine ratio determination (see ref. 10 for a simple procedure). Together with this latter determination, however, our system gives an unequivocal indication of the composition of a large majority of the miscellaneous GAG fractions we have had occasion to examine. Brief reference has been made to this electrophoresis method in a review article [11].

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