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Note

Fingerprinting of heparins by low-amperage electrophoresis in barium acetate

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Heparin, a polysaccharide widely distributed in animal tissues and well known for its anticoagulant and antilipaemic properties, has a hybrid structure, the major features of which are known. However, it has become increasingly evident that heparin is not a single species, but consists of a family of polysaccharide chains having a number of structural features in common, but differing from each other in minor detail and in molecular weight. Although polydispersity is readily demonstrable by gel filtration, the elution profiles obtained are usually broad and show no real separation of distinct entities (for recent reviews, see ref. 1)*.

The concept of structural heterogeneity has been reinforced by a number of observations in the past few years. Most heparin preparations give two spots on agarose[3] or cellulose acetate[4] electrophoresis in barbital buffers, and are separated into two fractions, one non-mobile and the other mobile, in a barium acetate buffer[5].

A much more pronounced degree of heterogeneity has been reported in isoelectrofocusing experiments, which show up to 21 bands for heparins that give a maximum of two spots in conventional electrophoretic systems[6]. However, this behaviour does not necessarily imply that such a large number of sub-fractions are present, because it has been shown that the carrier ampholytes involved in complexing are themselves preferentially localized in well defined zones of the isoelectrofocusing slabs[7]. Thus, multiple banding seems to reflect, at least in part, "saturation" of these ampholyte zones by excess of heparin as it moves towards the anode. Single heparin bands transferred on to new slabs produced either 1–3[8] or up to 21[7] bands, depending apparently on the concentration of the polysaccharide in the band transferred, thus not providing real information on the number of sub-fractions.

In an attempt to provide a simple criterion for evaluating the heterogeneity of heparins, we have extended the original observation by Wessler[5] that a portion of heparin does not migrate on cellulose acetate strips during electrophoresis in a barium acetate buffer. We now report that by working at lower amperage and temperature for longer times, electropherograms are obtained showing well defined bands for heparin preparations that, otherwise, are pure by any generally accepted criteria.

* A heparin preparation from placenta gave, however, distinctly separated sub-fractions from Sephadex G-50[2].

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MATERIALS AND METHODS

Heparins (sodium salts) were commercial samples that appeared to be electrophoretically homogeneous in an acidic buffer (0.05 *M* potassium chloride in 0.1 *M* hydrochloric acid, adjusted to pH 1.1, 4°C)[5,10] that permits the detection of admixed hyaluronic acid, heparan sulphates and galactosaminoglycans. Contamination by more than 3–4% of hyaluronic acid or galactosaminoglycans was also ruled out by the ^{13}C nuclear magnetic resonance spectra. A preparation from pig mucosa was a reference standard from the University of Chicago (Drs. A. J. Cifonelli and M. B. Mathews). The anticoagulant activity of the preparations was between 145 and 170 USP units.

The electrophoreses were performed in barium acetate (0.1 *M*) adjusted to pH 5.8[11] with acetic acid, on Sepraphore III (Gelman, Milan, Italy) or Microphor (Elvi, Milan, Italy) cellulose acetate strips (160 × 25 mm), at a voltage corresponding to 0.4 mA/cm, at 4°C for 16 h. Before application of the sample, the strips were immersed for 30 min in 0.1 *M* barium acetate (pH 5.8) and the excess of buffer was eliminated by blotting with a paper towel. Heparins were applied as sodium salts (1–3 μl of 0.2% solutions in water or 0.1 *M* barium acetate, pH 5.8). After application of the spots (or bands), the strips were left for 5 min on the electrophoresis bridge before applying the appropriate voltage. After staining with 1% Alcian Blue (Bio-Rad Labs., Richmond, CA, U.S.A.) in a 1:1 (v/v) mixture of 0.05 *M* sodium acetate and 95% ethanol, the strips were washed with 5% acetic acid and air-dried.

The densitometric traces were recorded with a Zeiss KM3 spectrophotometer, equipped with a reflectance unit set at 380 nm (slit 3.5 × 1.5 mm). As the colorimetric response to Alcian Blue was expected to be different for different heparin subfractions, the relative areas of the electrophoretic bands were not assumed to be a direct measure of the relative concentration of the sub-fractions.

Strict adherence to the above electrophoretic and densitometric conditions is required for obtaining reproducible electropherograms. It should be noted in particular that the electrophoretic patterns vary somewhat for different brands of commercial cellulose acetate electrophoresis products, and that the relative intensity of the various heparin bands as measured by reflectometry is different when scanning is made by transmission densitometry. The latter effect appears attributable to the non-mobile fractions being in fact "precipitated" on the cellulose acetate strips, and thus preferentially concentrated on the surface of the strips.

RESULTS AND DISCUSSION

The electropherograms of four heparin samples from typical commercial sources (bovine lung and pig intestinal mucosa) are compared in Fig. 1. One of the two bovine lung preparations (a) consists largely of a single component, which hardly moves from the origin. The second bovine lung heparin (b) clearly shows at least three more, fastest moving, components. Fast-moving components are represented much more extensively in mucosal heparins (c) and (d). Except for the profile of the band near the origin (broken lines), such traces are reasonably reproducible, and substantially different from each other for more than 20 heparin preparations so far analysed. These electropherograms can thus be regarded as "fingerprints" of heparins

that otherwise show only minor differences in their chromatographic and bulk physico-chemical properties.

The electrophoretic mobility of heparins in barium acetate seems, at least in part, to be a function of molecular weight. In fact, different fractions obtained by gel filtration (Ultrogel AcA-44) gave different electrophoretic profiles by the present method, with a general trend towards lowest mobility by material of the highest molecular weight, and *vice versa*. As shown in Fig. 2, intermediate fractions gave prominent peaks in the intermediate zone. On the other hand, heparins with similar profiles on gel filtration gave different electrophoretic patterns in the present system.

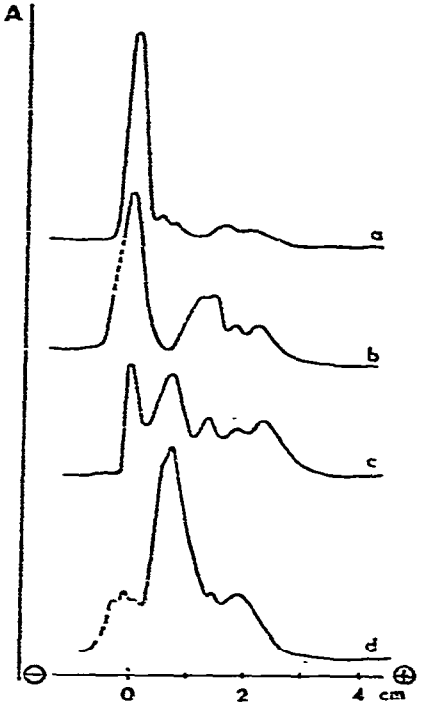

Fig. 1. Low-amperage electropherograms on cellulose acetate (0.1 *M* barium acetate, pH 5.8) of different heparin preparations: (a) from bovine lung (Upjohn, batch 070-ES); (b) from bovine lung (Upjohn, batch 746-DU); (c) from pig intestinal mucosa (Serva, batch 24590); (d) from pig intestinal mucosa (standard from the University of Chicago).

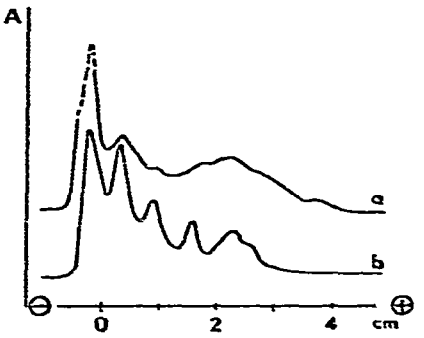

Fig. 2. Low-amperage electropherograms on cellulose acetate (0.1 *M* barium acetate, pH 5.8) of an unfractionated heparin [(a), from pig intestinal mucosa, Terhormon, batch 575-018] and an intermediate fraction obtained by gel filtration of the same heparin on Ultrogel AcA-44 (b).

Complexing with Ba^{2+} ions is likely to be the main factor influencing these separations, and it is concluded that the high-molecular-weight species complex Ba^{2+} ions stronger than do the low-molecular-weight species. This behaviour parallels that on isoelectrofocusing[6,8,12], where a trend is apparent in which high-molecular-weight fractions are more easily complexed by the carrier ampholytes than low-molecular-weight fractions.

The results show that "heparin" consists of a family of well differentiated species rather than a "continuum" of chains only barely distinguishable from each other. Although differences between these various species may prove to be relatively minor in terms of such criteria as overall composition, the number and/or arrangement of sites in them that determine their ability to complex with barium (and probably other divalent cations) must vary widely.

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