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THE MOLECULAR-WEIGHT RANGE OF MUCOSAL-HEPARIN PREPARATIONS

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ABSTRACT

A recently reported method describes the determination of the molecular-weight range of heparins by using an electrofocusing procedure to isolate individual molecular species. Commercially available heparins were fractionated on a column of polyacrylamide–agarose gel to give fractions whose molecular weights were estimated by viscometry. Fractions with mutually exclusive molecular-weight ranges gave an appreciable number of common bands when subjected to the electrofocusing procedure; therefore, each of these bands cannot be formed from a single molecular species of heparin. Other mucopolysaccharides also gave band sequences indistinguishable from those of heparin; they differed in position and intensity with different ampholyte batches, and probably arose from individual molecular species of the ampholyte rather than the mucopolysaccharide. The molecular-weight range of the heparin was observed to be broader than that usually reported.

INTRODUCTION

For many years, it has been recognised that "heparin" is highly polydisperse. Preparations are often now reported[1] to have a molecular-weight range between ~6000 and 20,000. Reports on the relation of anticoagulant activity *in vitro* to molecular weight have not been altogether consistent, but there is some agreement that, even though there may be no great variation over most of the range, it decreases in the region of lowest molecular weight[2,3]. However, a recent study[4] has shown that, irrespective of the results of assays *in vitro*, there are marked differences *in vivo* between fractions of high and low molecular weight from the same batch of heparin under certain, clinically significant conditions. A further examination of the molecular-weight range of commercially available heparins therefore seems justified, especially as a recent paper[5] reported a simple method of isolating and estimating individual molecular species of heparin by an electrofocusing procedure.

Batches of commercial mucosal-heparin were separated by gel filtration into fractions. Viscosity measurements were made on eight of these, and their molecular-weight distributions recorded on smaller columns of two different gels. The behaviour

of five fractions on electrofocusing was compared with that of desulphated and resulphated heparins and of other mucopolysaccharides.

MATERIALS AND METHODS

Commercial mucosal-heparin was kindly supplied by Evans Biologicals Ltd., Paines and Byrne Ltd., and Weddel Pharmaceuticals Ltd. There were some differences in molecular-weight distribution between samples, but in other respects there seems to be little to distinguish the heparins from these suppliers in the U.K.; much of the heparin is imported from a single source (see Fig. 1).

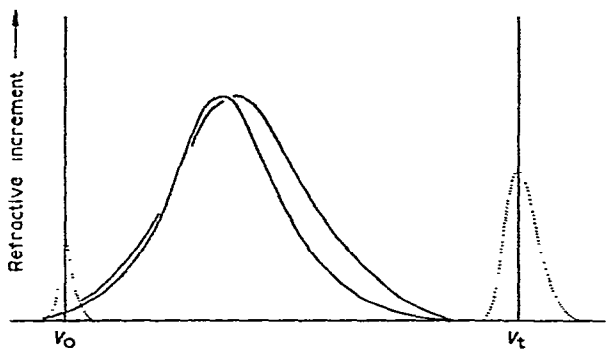

Fig. 1. Chromatography on Ultrogel AcA44 of two batches of commercial mucosal-heparin from the same manufacturer. Most U.K. heparin appears to fall within these limits. The dotted peaks are of Blue Dextran and excess sodium chloride, and indicate resolution; $V_0 = 9.2$ ml, $V_t = 27.8$ ml.

Professor J. A. Cifonelli (University of Chicago) kindly donated five heparin fractions, with narrow ranges of molecular weight, which had been resulphated after partial desulphation. These fractions are identical to those referred to as I-S^b,II-S^b, III-S^b, and IV-S^b in Ref. 6, except that the original IV-S^b had been subsequently fractionated into portions of higher and lower molecular weight, here termed IV-S^b and V-S^b, respectively.

Dr. Helen Muir (Kennedy Institute of Rheumatology, London) kindly provided samples of sulphated mucopolysaccharides of different origins: *HM1* is cartilage chondroitin sulphate; *HM2* is a mixture from aorta containing chondroitin and dermatan sulphates with lesser proportions of heparan sulphate and hyaluronic acid; *HM3* is urinary material from a patient with Hurler's Syndrome, containing dermatan sulphate, some heparan sulphate, and a little chondroitin sulphate; *HM4* is as *HM3*, enriched in heparan sulphate by electrophoresis; *HM5* is as *HM2*, chromatographically enriched in dermatan sulphate. Shark chondroitin sulphate was obtained from Koch–Light Ltd., Colnbrook, England.

N-Desulphated heparin was prepared by allowing a solution of heparin in the H^+ form to autolyse for 21 days at room temperature, and then neutralising with

sodium hydroxide. By this procedure, ~75% of the hydrolysable *N*-sulphate groups are removed and the anticoagulant activity is reduced to less than 10 u/mg.

Fractionation of the heparin was carried out on a column (109 × 2.8 cm) of Ultrogel AcA44 agarose–acrylamide gel (LKB-Produkter AB). For recording molecular-weight distributions, a column (58 × 0.75 cm) of Ultrogel AcA44 was used. As solvent and eluent, 0.3M NaCl containing 0.01% of NaN_3 was used, with flow rates of 48 and 4–6 ml/h for the preparative and analytical columns, respectively. Sample loads were ~0.5 g and 1.5 mg, respectively; for the preparative column, loads were dissolved in 12–20 times their weight of solvent to keep the viscosity fairly low. Differential refractometers (Waters Associates R403 and R401) were used as chromatographic detectors. The positions of the maxima of the distribution curves so obtained are given as values of $K_{av} = (V_{max} - V_0)/(V_t - V_0)$, where V_0 is the void volume (the elution volume for Blue Dextran 2000, LKB), V_t the total volume (the elution volume for excess NaCl introduced with the sample), and V_{max} the elution volume for the sample maximum[7]. The analytical columns were characterised by determining K_{av} values for two proteins, namely myoglobin (*M*), mol. wt. 17,000, and bovine serum albumin (*A*), mol. wt. 69,000. These molecular weight values bear no necessary relation to those of heparin fractions eluting at similar K_{av} values[8,9]; the elution positions for the proteins are marked to enable the performance of other similar columns to be compared.

For electrofocusing, the LKB Multiphor equipment was used, with gel slabs (24 × 10 cm) containing 2% of ampholyte. LKB Ampholines of various ranges were used, with Alcian Blue as the stain.

Viscosities were determined in 0.5M NaCl at 25° using a suspended level viscometer with a flow time for the solvent of 4.68 min. The samples were dried *in vacuo* (<0.1 torr) over P_2O_5 at room temperature for at least 16 h, and initial concentrations of between 0.6 and 0.9% w/v were used. The values of $\eta = [(t_c/t_0) - 1]/c$, where c = concentration, t_c = solution flow-time, and t_0 = solvent flow-time, were almost independent of c for concentrations of this order and lower (see also Laurent[10]).

Anticoagulant activities of the fractions were determined by a partial thromboplastin time method.

RESULTS

Molecular-weight distribution curves for two typical, commercial samples of heparin on the analytical Ultrogel column are given in Fig. 1. Under the heavy loading conditions used with the preparative column, the elution patterns did not match those obtained with small loads on an analytical column. It was therefore difficult to match fractions from runs using different starting materials; even after some mixing and re-running, about fifteen fractions differing significantly in their K_{av} values were obtained. Of these, eight, distributed across the range, were selected as available in amounts sufficient for viscometry. Their distribution curves on the analytical column are given in Fig. 2, and a plot of limiting viscosity against K_{av} in Fig. 3.

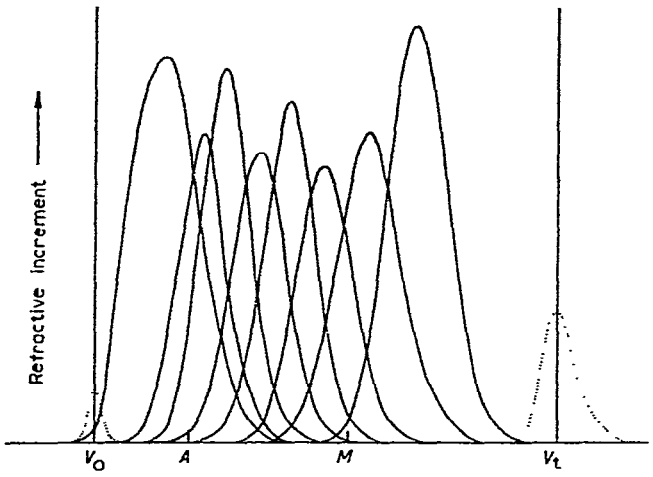

Fig. 2. Chromatography of Fractions 1–8, reading from left to right. *A* and *M* mark peak positions of bovine serum albumin and myoglobin. Conditions as for Fig. 1.

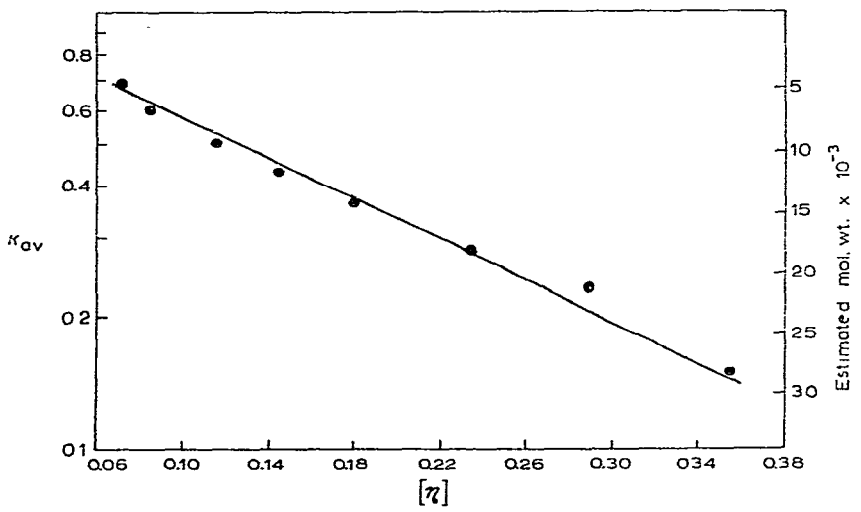

Fig. 3. The relation between intrinsic viscosity and gel-filtration elution volume on Ultrogel AcA44. The estimated molecular-weight scale applies to the line and not to individual points.

Table I gives properties of the heparin fractions. Plates 1 and 2 show the results of electrofocusing using, respectively, 2% of Ampholine pH 3–6 (Batch No. 26), and a mixture (2% total) of this material with pH 2.5–4 (Batch No. 7), pH 4–6 (Batch No. 17), and pH 5–8 (Batch No. 43) in the ratios 5:2:4:4. When using a mixture of the last three Ampholines alone, difficulty was experienced from overheating in a zone of low conductivity, but continuity of bands along the length of the gel could be seen, as on Plates 1 and 2, for all the mucopolysaccharides.

TABLE I

DATA ON HEPARIN FRACTIONS

Fraction no.	K_{av}	$[\eta]$	*Estimated mol. wt.*		*Anticoagulant activity (u/mg)*	
1	0.15	0.355	29,000		106	
2	0.23	0.288	23,000		157	
3	0.28	0.235	18,500		170	
4	0.36	0.180	14,000		187	
5	0.43	0.145	11,000		111	
6	0.50	0.113	8,200		80	
7	0.60	0.084	5,900		30	
8	0.69	0.072	5,000		5	
I-S[b]	0.18	—	23,000[a]	14,000[b]	250,	153[b]
II-S[b]	0.285	—	17,000[a]	10,500[b]	200,	156[b]
III-S[b]	0.395	—	11,000[a]	7,900[b]	168,	162[b]
IV-S[b]	0.50	—	7,600[a]	6,500[b]	73	92[b]
V-S[b]	0.625	—	5,300[a]		20	

[a]Using data points from Mathews (see text). [b]Values from Cifonelli[6].

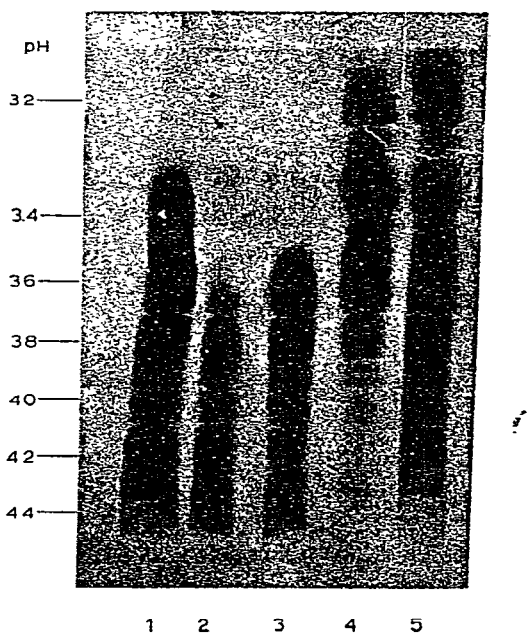

Plate 1. Electrofocusing on gel containing 2% of Ampholine, pH 3–6: 1, *N*-desulphated heparin; 2, Cifonelli Fraction I-S[b]; 3, Cifonelli Fraction III-S[b]; 4, Cifonelli Fraction V-S[b]; 5, unfractionated heparin.

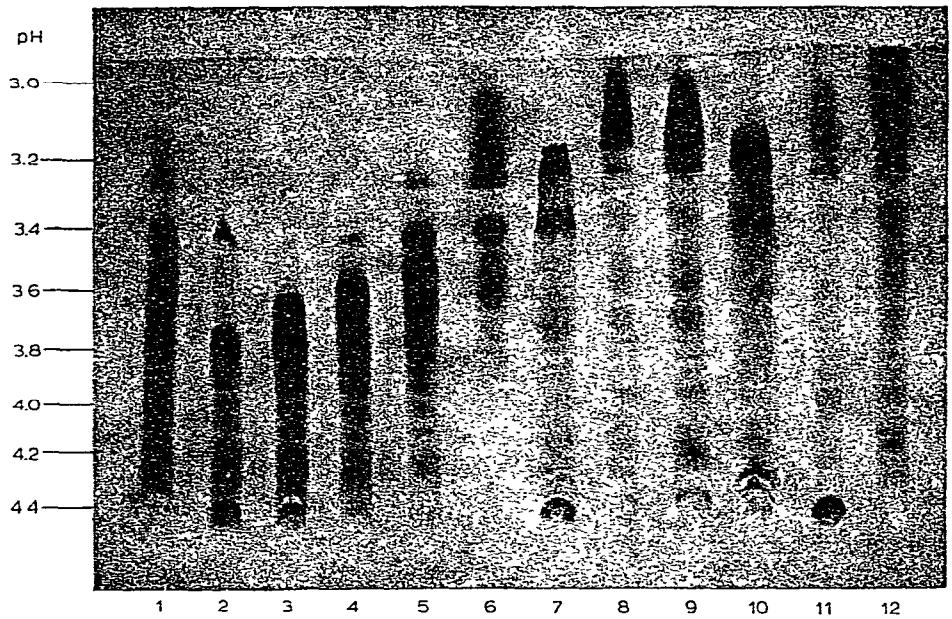

Plate 2. Electrofocusing on gel containing mixed Ampholines (see text): 1, unfractionated heparin; 2, Fraction 1; 3, Fraction 3; 4, Fraction 4a (similar to Fraction 4, with K_{av} on UG44 of 0.38); 5, Fraction 6; 6, Fraction 8; 7, shark chondroitin sulphate; 8, cartilage CS (*HM1*); 9, aorta mucopolysaccharides (*HM5*); 10, aorta mucopolysaccharides (*HM2*); 11, urine mucopolysaccharides (*HM3*); 12, urine mucopolysaccharides (*HM4*).

DISCUSSION

Heparin has been fractionated by gel-filtration methods by a number of workers[2,8,9,11], but there has previously been no report of an examination of the molecular-weight range of commercial mucosal-heparin by fractionation of this kind followed by the estimation of the molecular weights of isolated fractions by generally applicable methods.

Although equilibrium sedimentation is probably the best method available for determining the mean molecular weights of heparins, there is a useful body of literature correlating measurements of this kind with limiting viscosity (Fig. 4). The equation relating limiting viscosity $[\eta]$ to molecular weight may be expressed in the form $[\eta] = K\mathrm{M}^{\alpha}$, where K is a constant, M the molecular weight, and the exponent α may vary between 0.8 and 1.0, depending on ionic strength and pH (see Lasker and Stivala[3]; over most of the range, the effect of pH change is likely to be small, and even below pH 3 the conversion of $-COO^-$ into $-COOH$ is unlikely to make much difference in the presence of all the surviving $-SO_3^-$ groups). Although the available literature data[3,10,12,14] vary in both these respects, there is reasonable concordance between plots of limiting viscosity against molecular weight determined by sedimentation methods (Fig. 4).

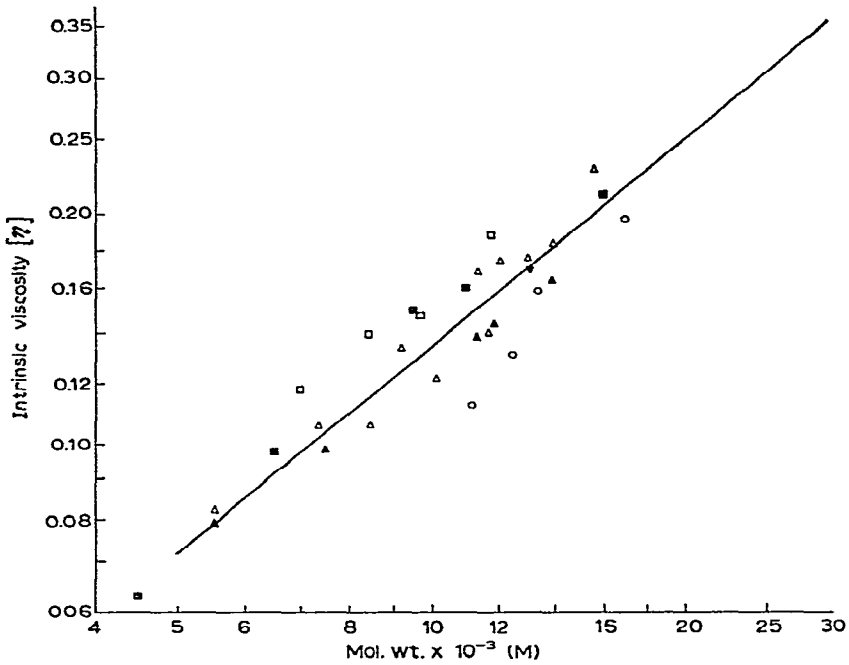

Fig. 4. The relation between intrinsic viscosity and molecular weight, as determined by sedimentation methods, obtained by previous workers. The line is plotted from the equation $[\eta] = 1.35 \times 10^{-5} M^{0.9}$, and falls between the results of Mathews and of Lasker and Stivala in the same solvent (0.5 M NaCl): □ Laurent[10], 0.1M NaCl; ■ Mathews (data from Cifonelli), 0.5M NaCl; △ Lasker and Stivala[3], 0.1M NaCl; ▲ *ibid.*, 0.5M NaCl; ▼ Stivala[14], 0.5M NaCl; ○ Braswell[12], M NaCl.

Two factors (apart from technical difficulties in measurement) may play a part in the scatter shown by points at constant ionic strength. One is the procedures used to determine and to calculate the sedimentation data (see Braswell[12]), and the other is the method of fractionation. Lasker and Stivala[3] derived somewhat different lines from fractions separated by chromatography on ECTEOLA cellulose and from fractions precipitated by increasing concentrations of ethanol. Laurent[10] used a cetyl pyridinium chloride fractionation method. It is likely that each of these procedures will produce fractions that differ in polydispersity and also in such physicochemical properties as charge density and consequent size of the counter-ion shell in solution. Such differences may affect viscosity, sedimentation, and gel-filtration behaviour to varying extents, and hence alter the exponent α in the limiting-viscosity equation given above. It is difficult to question the breadth of the molecular-weight range observed in the present work unless some reason is found for postulating that this exponent can be appreciably greater than 1.0. The estimated weights given in Table I are taken from the line shown in Fig. 3, for $[\eta] = 1.35 \times 10^{-5} M^{0.9}$, except for those for the fractions provided by Professor Cifonelli, which are somewhat reduced to fit a best line through the data points of Mathews, also supplied by him.

The relationship between K_{av} and viscosity or molecular weight shown in Fig. 3 was plotted from results on a fairly new column. Although Ultrogel gives columns having excellent stability and flow characteristics which can be used for a great number of runs, the K_{av} values decrease somewhat with time. At the high end of the molecular-weight range, Ultrogel AcA34 becomes more suitable.

Cifonelli's results on his fractions in the high molecular-weight region show large variations from the values found in the present work (Table I). Different gels were used; although we have not found Sephadex G75 to be very suitable for heparin fractionation, and even with G100 resolution at the high molecular-weight end is not very good, the degree of polydispersity of both the Cifonelli fractions and our own is much the same. Also, our first indication that the molecular-weight range of commercial heparins might be broader than is generally acknowledged[4] came from fractionation on G100. Their distributions are invariably of the wide and symmetrical kind shown in Fig. 1, and a comparison with Fig. 2 shows that the content of material at the ends of the range, *i.e.*, Fractions 1 and 8, is not negligible.

The claim that individual molecular species of heparin are separated by electrofocusing is not tenable, as may be seen by comparing Fig. 2 and Plate 2. If allowance is made for diffusion-broadening of the peaks in Fig. 2, it is clear that, for example, there is almost no material common to Fractions 1 (or even 3) and 6. On Plate 2, there are many bands common to these fractions, and the overlap is considerable in terms of total stainable material. The same is true, on Plate 1, for fractions I-S^b and V-S^b, which, by gel chromatography, also have no material in common. The general tendency for material of lower molecular weight to run to the low-pH end of the gradient is clear, but factors other than molecular weight must determine precipitation at any particular point. In both Plates, the horizontal continuity of the bands across each gel is independent of the nature of the mucopolysaccharide (this was much clearer on the original gel), and they must be identified as arising from components, perhaps individual molecular species, of the ampholyte. There is, therefore, nothing to suggest that the spectrum of heparin molecular weights is other than a continuous one, except in so far as the continuity must be limited by the requirements of the synthetic process, as yet imperfectly understood.

The anticoagulant activities, as measured by the partial thromboplastin-time procedure, show a marked drop at the low molecular-weight end, and also (except for Fraction I-S^b) show a smaller but distinct drop at the high molecular-weight end. Kavanagh and Jaques[13] found no useful correlations between any of the chemical parameters usually determined and the biological activities for eighteen commercial heparins. The intention of the present work was to demonstrate clearly the molecular-weight range present in an ordinary, commercial heparin, typical of the fairly standard, imported product extensively used in the United Kingdom, and there is no reason to think that attempts to correlate analytical data on the fractions with activity will meet with better success. Further work on the biological activities of the fractions is being undertaken.

ACKNOWLEDGMENTS

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