The potency of heparin fractions obtained by gel filtration

It is generally agreed that heparin is polydisperse in molecular weight, and there is evidence that blood anticoagulant activity increases with increasing molecular weight. Thus, Laurent (1961) precipitated the cetyl pyridinium salt of heparin in a series of fractions, and found the least soluble fraction to have the highest molecular weight, as measured in the ultracentrifuge, and also the highest anticoagulant activity. Successive fractions in decreasing order of molecular weight were of decreasing activity.

Gel filtration separates molecules according to their mean molecular size (Stokes radius). Ricketts, Walton & Bangham (1966) found that samples of heparin of higher potency contained more heparin molecules of larger size, as shown by the position of peaks, from a column of Sephadex G-100. It therefore seemed very likely that if heparin fractions from a Sephadex column could be assayed individually for anticoagulant activity, early fractions from the column containing larger molecules would show higher potency than later fractions.

Heparin of porcine mucosal origin* (50 mg/ml of solution), having a potency of 212 units/mg, was submitted to gel filtration on a column of Sephadex G-200 45 cm long by 2·5 cm diameter, in 0·15 M sodium chloride solution (9 vol), 0·12 M sodium phosphate buffer pH 7·4 (1 vol.) containing 0·05% sodium azide as preservative. The column was eluted at 11·55 ml/h into 5 ml fractions. The effluent solution was monitored by a differential refractometer (Waters Model R4). This provided a record of the difference in refractive index between solution entering and leaving the column, as shown in Fig. 1, curve I. Void volume and total volume were measured with blue dextran and sodium iodide respectively in a preliminary experiment; K_{av} was calculated from these and the elution rate. Previous experiments had shown that the recorder reading was proportional to the concentration of polysaccharide in

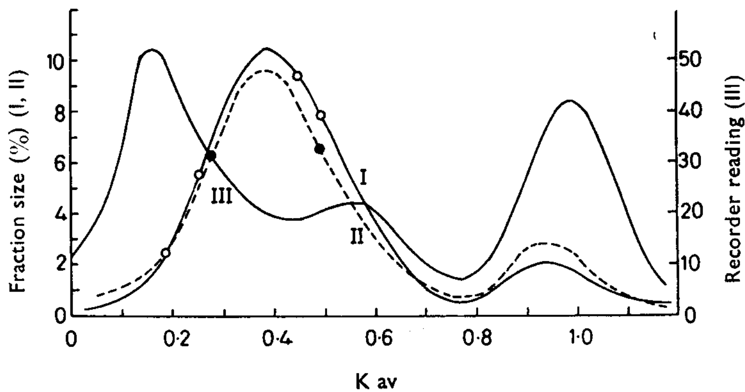

FIG. 1. Curve I. Gel filtration of pig mucosal heparin (212 units/mg) on a column of Sephadex G-200. Fraction size as percentage of whole sample calculated from refractive index change and plotted against K_{av} ($= V_e - V_o/V_t - V_o$ where V_t = total volume, V_e = elution volume and V_o = void volume of column). The heparin peak is at K_{av} 0·4; the peak at K_{av} 0·95 was due to inactive impurities. ○ Fractions selected for assay of anticoagulant activity.

Curve II. Gel filtration of a further sample of pig mucosal heparin (159 units/mg), details as above. Fractions with K_{av} value 0·27 and 0·49, symmetrically placed on each side of the peak, were selected for measurement of anticoagulant activity. ● Fractions selected for assay.

Curve III. Three fractions from each side of the heparin peak of Curve A having K_{av} values 0·14–0·21 and 0·60–0·68, were mixed and the mixture was separated on the same column of Sephadex G-200 into two heparin peaks with K_{av} values 0·16 and 0·56 showing that the gel filtration was separating molecules according to size and that the peak of Curve A was not simply due to diffusion. The third peak at K_{av} 1·0 is due to salts.

* Samples of high potency heparin were supplied by Weddel Pharmaceuticals Ltd., West Smithfield, E.C.1. where all assays were made.

Table 1. *Heparin fractions obtained by gel filtration*

Sample	Fraction No.	K_{av}	Weight (mg)	Activity (units)	Potency (units/mg)
	23	0·17	1·04	240	230
A	24	0·21	1·74	312	179
(212 units/mg)	30	0·45	4·65	1120	240
	31	0·49	3·87	700	180
B	26	0·27	2·86	540	188
(159 units/mg)	32	0·49	3·18	550	173

the solution. The percentage of the sample appearing in each fraction was calculated from the area under the curve. All fractions were freeze-dried and sealed in glass ampoules. Fractions selected from each side of the main peak were dissolved in distilled water and the number of units of anticoagulant activity present in each fraction was assayed.

The assays (B.P. 1963, p. 1136) were in terms of the "First British Standard Mucous Heparin" using the coagulometer described by Walton & Wright (1964). The methods for the determination of potency and calculation of variance were those of the B.P. 1963 (p. 1088).

Table 1 lists the fractions assayed, their K_{av} value, the number of units of anticoagulant activity found and the weight of heparin present, calculated from the refractive index recording. To minimize dependence on calculation of the weight of heparin in each fraction it was thought desirable to repeat the experiment selecting fractions having the same recorder reading. This was done using a further sample of mucosal heparin of potency 159 units/mg. The elution curve, Fig. 1, curve II, obtained closely resembled curve I, as indeed did the elution curves of several other samples of heparin of mucosal origin. Results of this second experiment are also given in Table 1. When the potency of the fractions investigated is plotted against their K_{av} value, taking all the results together there is no systematic variation with K_{av} values. Thus, the expectation that fractions of heparin containing molecules of greater molecular diameter would prove more potent has not been realized.

It remained to be shown that gel filtration under these conditions was in fact separating molecules reproducibly and that the almost symmetrical peak obtained was not simply due to diffusion. A group of fractions from the high molecular weight side of the peak of curve I in Fig. 1 was mixed with a group from the low molecular weight side of the peak. The solution was concentrated by ultrafiltration and dialysed against the buffer solution used for elution; 1 ml of the concentrate was applied to the Sephadex column and eluted as before. The elution curve, Fig. 1 III, showed two peaks at the appropriate K_{av} values due to heparin and a third peak due to salts. The two heparin peaks are not of equal height; it is possible that some of the smaller molecules were lost through the Cellophane tubing during ultrafiltration. It is believed therefore that under these experimental conditions heparin molecules were being separated according to their size.

The samples of mucous heparin used in this work were of relatively high potency, 159 and 212 units/mg. The experiments have shown that while this heparin is certainly polydisperse in molecular size it is relatively homogeneous in anticoagulant activity. It seems quite likely that a highly potent preparation of a natural product would be more homogeneous in respect of biological activity than a less potent sample, part of which may have undergone some small alteration of chemical structure during its isolation. This may very well be true of heparin samples where the exact relation between detail of chemical structure and biological activity remains incompletely understood.

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May 1, 1969

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A use of the isomeric ratio as a criterion to differentiate adrenergic receptors

The general concept of α- and β-adrenergic receptors is well recognized. Rossum (1965) indicated, however, that different tissues may contain different types of α-adrenergic receptors. Although this is a theoretical possibility, Furchgott (1967) found similar K_B values for phentolamine in three different tissues of the rabbit and, on this basis, suggested that the α-adrenergic receptors in thoracic aorta, muscle from the corpus of the stomach and duodenum of rabbit are of a single type.

As with other pharmacologic receptors, a very characteristic property of adrenergic receptors is that of stereoselectivity. If α-adrenergic receptors in various tissues are of a single type, and if their ability to interact with optical isomers is stereoselective, it follows that the activity difference or the isomeric ratio between (−) and (+)-noradrenaline should be the same in each tissue. Under normal circumstances, this isomeric ratio is obscured by several factors operative at the adrenergic nerve terminals; the stereoselective uptake, the unequal distribution of antagonistically-acting α- and β-adrenergic receptors in the same tissue, and the presence of enzymes which can cause selective degradation of isomers of noradrenaline. If all these factors were properly controlled, it is possible that the isomeric ratios of noradrenaline in different tissues containing α-adrenergic receptors would be identical. Data in Table 1 were readily available from previous reports from ours and other laboratories. It can be seen that in normal tissues, the isomeric ratios vary from 2 to 64 (a 32 fold variation). Since acute treatment with reserpine does not significantly change the neuronal uptake, it did not change the isomeric ratio. However, if neuronal uptake was inhibited by cocaine or a cocaine-like agent, imipramine, the isomeric ratios were markedly altered. For cat blood pressure, nictitating membrane, spleen and rat vas deferens, the isomeric ratios for noradrenaline in the presence of cocaine or cocaine-like agents only ranges between 50 and 80. These ratios, within the limits of experimental error, may be considered as essentially equal. Rabbit jejunum has a low density of adrenergic innervation (0·5 μg/g). This is reflected in a high isomeric ratio in the normal tissue. In other words, after inhibiting the uptake, this isomeric ratio of 64 may not change significantly. Similarly, in the rabbit aorta, due to low adrenergic innervation relatively high isomeric ratio was obtained. Thus, the isomeric ratios in all these six tissues which mainly contain α-adrenergic receptors, there is a tendency for isomeric ratios to be equal. It varies from 42 to 80 (*i.e.*,