THE CHARACTERISATION OF HEPARIN IN BOVINE LIVER CAPSULE

A. SERAFINI-FRACASSINI, C.J. BRANFORD WHITE and J.C. HUNTER
Department of Biochemistry, University of St. Andrews, St. Andrews, Fife, Scotland

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1. Introduction

The molecular organisation of heparin in bovine liver capsule is still under debate. Other sulphated glycosaminoglycans present in connective tissue matrices are covalently bound to proteins and form multichain complexes. Moreover, their protein cores act as specific acceptors for the initiation of biosynthesis. As reported by Silbert [1], heparin also requires a protein primer and therefore should exist in the mast-cell granule as a proteoheparin complex [2]. In a previous paper it has been reported that the proteoheparin of liver capsule has a protein content of about 13% and a molecular weight of 20,000 daltons [3, 4]. Contrary to these findings, Lindahl has identified protein-free chains of 7,400 mol.wt. as the major heparin species present in this tissue [5]. In order to clarify this position, we have investigated the variability in composition and molecular weight of heparin isolated by a dissociative extraction procedure, minimizing the possibility of physical macromolecular disruption.

2. Experimental and results

Bovine liver capsule was stripped free from liver tissue and powdered after treatment with acetone. Portions (50 g each) of this material were extracted twice with 0.1 M citrate, pH 3.1, to remove soluble proteins. It is considered that under the above conditions of ionic strength and pH, heparin set free into the matrix, as a result of the disruption of the mast-cell granules, would bind to collagen [6] and consequently would not be released into the extraction medium. After centrifugation, the residue was collected and extracted by gentle stirring with unbuffered 3 M $MgCl_2$ for 48 hr. The extract was collected and dialysed against water. A precipitate that formed during dialysis was separated by centrifugation and stored (fraction H1). Three volumes of ethanol and one volume of ethanol saturated with K-acetate were added to the supernatant and the precipitated material was collected (fraction H2). The entire isolation procedure was carried out at 4° in order to minimize protease and heparinase activities [7–9].

Fractions H1 and H2 were dissolved in 0.4 M citrate, pH 5.0, and chromatographed on Whatman DE-52 Cellulose by step-wise elution with 0.1 M citrate, pH 4.0, containing varying concentrations of NaCl (0.25 M increments were used). Fractions were tested for hexuronic acid [10] and commercial heparin (Wilson Laboratories, Lot No. 136651) was used as a standard. In the case of H1, hexuronic acid-containing material was present only in the 0.75 M eluate. On the other hand, H2 yielded two hexuronic acid-containing fractions which were eluted at 0.5 M NaCl (fraction H2a) and at 0.75 M NaCl (fraction H2b).

The three fractions were subsequently dialysed against 0.01 M citrate, pH 5.0, and chromatographed on Sephadex C-50. In each case the excluded material, which contained the total amount of hexuronic acid, was dialysed against water and the glycosaminoglycans precipitated by the addition of ethanol and K-acetate at 4°. Homogeneity of the preparations was checked by gel filtration on Sephadex G-200 in 2 M KCl. Fractions H1 and H2b behaved as monodisperse species. On the other hand fraction H2a revealed the presence of at least three subfractions. Two of these had $GlcNH_2$/$GalNH_2$ ratios of 0.25 and 0.17 which indicated the presence in fraction H2a of galactosamine-containing glycosaminoglycans in addition to heparin. The third subfraction had a $GlcNH_2/GalNH_2$ ratio of 8.2 and will be referred to as fraction H2a*.

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Table 1
Analytical results on heparin fractions isolated from bovine liver capsule.

	H1	H2a*	H2b
Glucosamine	20.0	20.6	18.4
Galactosamine	0.0	2.5	0.5
Hexuronate (K salt)	29.7	34.3	35.4
Sulphate (K salt)	34.9	29.5	30.7
Galactose	3.1	3.5	2.9
Xylose	1.7	1.8	1.4
Protein	10.7	7.5	19.7
$S/GleNH_2$ (molar ratio)	2.6	2.2	2.5
$M_{n,app}$ (at meniscus) $\times 10^{-3}$	15.5	7.6	16.2
$M_{n,app}$ (at column base) $\times 10^{-3}$	22.0	13.6	20.2
Chain weight (calculated on the basis of xylose content) $\times 10^{-3}$	8.0	6.7	8.6
Biological activity (B.P. units/mg)	145	110	180

The yields of H1, H2a* and H2b were estimated to be approx. 15, 45 and 80 mg/100 g of dry tissue, respectively, although losses might have occurred during fractionation.

The compositions of H1, H2a* and H2b are reported in table 1 together with molecular weight and biological activity data. Protein contents were calculated from total amino acid concentrations corrected for hydrolytic losses. Concentrations of glucosamine and galactosamine were determined after chromatographic separation and neutral sugars were identified and quantitatively estimated by gas chromatography [11]. Sulphur was determined by two procedures [12, 13] and the concentrations reported are mean values. Molecular weights were determined by equilibrium sedimentation [14] on a Spinco Model E ultracentrifuge at 20° and at a rotor speed of 39,460 rpm. A partial specific volume of 0.56 ml/g was estimated by the micro-drop method [15, 16]. Anticoagulant activities were determined by two procedures [17, 18] and mean values are reported.

3. Discussion

Data reported in table 1 show that heparin species present in bovine liver capsule fall into two classes of macromolecules which exhibit a 1:2 relationship regarding their molecular weights. It should be noted that the range of $M_{n,app}$ obtained at vanishing concentration and at the base of the column for H1 and H2b may reflect either macromolecular aggregation or some degree of polydispersity of the glycosaminoglycan chains or both. The higher-molecular weight species, which constitute about 65% of the total heparin extracted, have a higher protein content than the remainder. However, other compositional data are largely comparable in the two classes, except for some variability in the degree of sulphation which may also explain differences in their biological activities.

The identification of galactose and xylose, in all fractions in a molar ratio which is in keeping with the reported structure of the linkage region [19], indicates that the potentially reducing ends of the glycosaminoglycan chains are intact in the tissue. Therefore, the lack of xylose reported by Lindahl in most of his isolated chains [5] must reflect degradation occurring during isolation.

The presence of an intact linkage region is suggestive of the presence of complexes in which heparin chains are covelently bound to a protein moiety. The chemical demonstration of such covalent linkages in H2b is at present under investigation. However, it is worth noting that the molecular weights at $c = 0$ of both H1 and H2b, as determined by equilibrium ultracentrifugation, are twice the number-average chain weight of the preparations, as calculated from xylose concentrations. This strongly supports the view that heparin species comprising these two fractions are constituted by two glycosaminoglycan chains joined by the protein moiety. The validity of this correlation is supported by the observation that chain weight and minimum molecular weight of H2a* are identical, when allowance is made for the contribution of the protein moiety to the molecular weight.

It is impossible to assess at present whether the single-chain heparin species, which constitute a large proportion of the molecules in H2a*, represent naturally-occurring macromolecules or degradation products, resulting from endopeptidase depolymerisation of the protein moiety of larger complexes either *in vivo* or during extraction.

In conclusion, these results clearly indicate that the majority of the heparin in liver capsule is in the form

of macromolecular complexes consisting of protein and two glycosaminoglycan chains. It is of interest to note that this structural organisation is remarkably similar to that found in proteochondroitin sulphate where glycosaminoglycan doublets are uniformly distributed along the axis of the macromolecule [20].

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