Short Communications

Isolation of a Heparin-Protein Complex from Ox Liver Capsule

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Although the localization of heparin within the mast-cell granule has been established (Hagen, Barnett & Lee, 1959), its organization *in vivo* has yet to be fully elucidated.

In a previous attempt to isolate the native heparin-protein complex, Julén, Snellman & Sylvén (1950) extracted ox liver capsule with phosphate buffer. The supernatant of this extract, after centrifugation at 60 000g, yielded a material that contained protein and lipid, in addition to the glycosaminoglycan. This lipoprotein complex, however, dissociated in the presence of saturated phosphate buffer, indicating the absence of covalent linkage (Snellman, Sylvén & Julén, 1951). Although Korn (1959) considered this complex to be an artifact due to the affinity of heparin for lipoproteins, during his study of heparin isolated from mast-cell tumours he could not find any evidence to support the covalent linkage of heparin to proteins.

On the other hand, Green, Day & Roberts (1961) were able to detect the presence of residual amino acids in heparin hydrolysates. Moreover, amino acid analysis of commercial heparin preparations (isolated mainly from pig tissues by enzymic treatment) revealed the presence of residual peptides in which serine predominated (Lindahl, Cifonelli, Lindahl & Rodén, 1965). The isolation of xylosylserine, after mild acid hydrolysis of these preparations, suggested that heparin probably occurs in the native state covalently bound to a protein (Lindahl & Rodén, 1965).

The present paper reports a new extraction technique based on the solubility properties of sulphated polysaccharide-cetylpyridinium complexes (Scott, 1960).

Defatted and dried ox liver capsule was extracted with 1% (w/v) cetylpyridinium chloride in 2M-KCl (20 ml./g.) at 50° for 1 hr. After removal of the sediment by centrifugation, the cleared supernatant was diluted to a final KCl concentration of 0.9M. The precipitate that formed was collected by filtration and redissolved in 2M-KCl. This solution was then shaken with chloroform-pentan-1-ol (5:4, v/v) to dissociate the cetylpyridinium from

the complex and remove it. The aqueous phase was dialysed exhaustively against water. After the addition of 4 vol. of ethanol, the solution was centrifuged at 38 000g_{av} for 1 hr., and the heparin-protein complex was precipitated by the further addition of 1 vol. of ethanol saturated with potassium acetate.

This procedure has the advantage of not requiring the use of enzymes or alkali, which would either degrade the protein moiety to peptides or remove it from the heparin by β -elimination. Moreover, the blocking of sulphate groups by cetylpyridinium ions prevents the interaction of the polysaccharide moiety of the complex with insoluble collagen during extraction and allows fractionation of the material from other mucopolysaccharides and DNA present in the extraction medium. It has also been reported that the chloroform-pentan-1-ol mixture, used to dissociate and remove the cetylpyridinium ion, will precipitate any protein not covalently bound to polysaccharide (Blix & Snellman, 1945). This stage therefore provides further purification of the heparin-protein complex from any residual contamination by proteins or peptides that are not inherent in the complex.

The protein content of the isolated material was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with the protein moiety of a chondromucoprotein preparation (Serafini-Fracassini, Peters & Floreani, 1967) as a standard to compensate for any interference due to the glycosaminoglycan. A value of approx. 12.9% was found. The hydrolysis for hexosamine estimation was carried out for 10 hr. in 4N-HCl (2 ml./mg.) at 100° in sealed tubes under N₂. A total hexosamine content of 14.8% was determined by the method of Cessi & Piliego (1960) against a glucosamine standard, although chromatographic analysis showed trace amounts of galactosamine. The hexuronic acid was estimated by the modified carbazole method of Bitter & Muir (1962) to comprise 27.83% of the complex. The sulphur content, determined by the method of Giellman & Tölg (1960), averaged 8.9%.

Comparison of the results of these analyses with

those previously reported for the light fraction of bovine chondromucoprotein (Partridge, Whiting & Davis, 1965; Buddecke, Kröz & Lanka, 1963; Serafini-Fracassini *et al.* 1967) shows that in both cases the protein moieties account for a similar proportion of the total complex.

Our values for sulphur, glucosamine and glucuronic acid, as percentages of the polysaccharide, show good agreement with those reported by Lindahl *et al.* (1965) for purified heparin fractions. Further, the sulphur content of our preparation, 11.0% of the polysaccharide, indicates that the isolation procedure causes very little, if any, damage to the glycosaminoglycan.

To evaluate the homogeneity of the preparation, it was dissolved in 0.1M-sodium phosphate buffer, pH 7.0, containing 0.4M-NaCl and submitted to analytical ultracentrifugation at 59780 rev./min. In this system the complex behaved as a single peak characterized by a sedimentation coefficient of 1.85s (Fig. 1).

Separation of a mixture of commercial chondroitin sulphate and commercial heparin was performed by column chromatography on aminoethylcellulose, by applying a stepwise gradient of NaCl in 0.01M-HCl. Chondroitin sulphate was eluted by 1.0M-NaCl, whereas heparin required 1.5M-NaCl. When the heparin-protein complex was chromatographed, concentrations of NaCl in

excess of 3M failed to displace it. However, after separation from the protein moiety by β -elimination with 0.2M-NaOH at 37° for 24hr. (Schienthal & Schubert, 1963), the polysaccharide was eluted by 1.5M-NaCl.

The controversy that has surrounded the nature of the native heparin-protein complex has undoubtedly been due to the absence of a satisfactory isolation procedure. This has resulted in the various types of complex reported by different authors in the past, most of which were probably due to the affinity of heparin for lipoproteins and other contaminants. However, the chemical analyses, the ultracentrifugation characteristics and the behaviour during aminoethylcellulose chromatography of the material extracted by the present technique indicates that heparin exists as a macromolecular complex with protein through covalent linkage, similar to other mucopolysaccharide-protein complexes already characterized.

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Fig. 1. Ultracentrifugal pattern of the heparin-protein complex. Its total concentration was 0.945% (w/v). The picture was taken at a schlieren angle of 60° at 80 min. after the rotor had reached a nominal speed of 59780 rev./min. The direction of sedimentation is from left to right.