Comment on the Use of Cetylpyridinium Chloride in the Isolation of Connective-Tissue Proteoglycan

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In this paper the term 'proteoglycan' refers to compounds in which a protein moiety is covalently bound to several sulphated glycosaminoglycan chains (Jeanloz, 1960).

The physiological properties of connective tissue largely depend on the presence of macromolecular proteoglycans, a group of compounds that has attracted considerable attention in recent years. The various methods used for the isolation of proteoglycans invariably involve extraction of tissues with water or salt solutions to yield mixtures of proteoglycans, proteins, glycoproteins and other macromolecules. The protein moieties of proteoglycans generally account for only minor portions of the entire molecules, and any attempt at their characterization therefore requires the complete removal of non-covalently bound proteins and glycoproteins.

Glycosaminoglycans can be purified by precipitation with quaternary amino compounds, e.g. CPC* or cetyltrimethylammonium bromide. This method, as developed by Scott (1960), has proved to be one of the most convenient means of isolating and fractionating glycosaminoglycans obtained from proteolytically degraded tissues.

The use of quaternary amino compounds has also been extended to include the isolation of proteoglycans. Owing to the difference in anionic charge density between proteoglycans on the one hand and proteins and glycoproteins on the other, fractionation is generally achieved by precipitation of proteoglycan with CPC or cetyltrimethylammonium bromide from solutions of adequate ionic strength. The separation obtained is usually quite clean, and it is therefore often assumed that any protein present in proteoglycan fractions after repeated precipitation is covalently bound to polysaccharide. However, the results now described demonstrate that proteoglycans prepared by repeated precipitation with CPC from solutions of high ionic strength might indeed contain contaminating protein in amounts greatly exceeding the amount of protein that is covalently bound to polysaccharide chains.

Attempts have been made to isolate proteoglycans of heparin from mouse mastocytoma and from bovine liver capsule (U. Lindahl, unpublished work). Extracts (2M-KCl) of the homogenized tissues were

mixed with equal volumes of 1% (w/v) CPC, and polysaccharides were precipitated by dilution to 0.8M-KCl at pH 9.0. The solution obtained on stirring the precipitate in 2M-KCl was clarified by passage through Celite and precipitation with CPC was repeated. The polysaccharides were converted into sodium salts (Lindahl, Cifonelli, Lindahl & Rodén, 1965), digested with hyaluronidase to remove chondroitin sulphates and twice reprecipitated with CPC.

The hyaluronidase-digested mastocytoma heparin contained one serine for every seven glucosamine residues; the total amounts of protein were consistent with the presence of a proteoglycan composed of several heparin chains linked to a single polypeptide. However, by repeated freezing and thawing of this preparation, followed by removal of insoluble material, the glucosamine/serine molar ratio was increased to 75:1, indicating the separation of a component consisting mainly of protein.

Table 1. *Amino acid analyses of CPC-precipitable proteins from mouse mastocytoma (A) and bovine liver capsule (B)*

Samples were hydrolysed *in vacuo* with 6M-HCl at 110° for 24 hr. Results are expressed as residues/100 residues.

	Preparation A	Preparation B
Aspartic acid	10.6	10.3
Threonine	5.7	4.8
Serine	7.0	8.1
Glutamic acid	12.8	11.3
Proline	7.1	7.5
Glycine	6.9	7.7
Alanine	8.1	9.0
Cystine (half)	1.4	0.7
Valine	6.2	5.3
Methionine	1.8	1.6
Isoleucine	4.5	4.9
Leucine	9.0	10.6
Tyrosine	2.9	2.3
Phenylalanine	4.1	3.5
Lysine	5.9	6.0
Histidine	1.8	2.3
Arginine	4.4	4.3
Glucosamine	—	3.6
Galactosamine	—	4.3

* Abbreviation: CPC, cetylpyridinium chloride.

Further studies demonstrated that heparin isolated from either mastocytoma or liver capsule occurred essentially as single polysaccharide chains, a proportion of which were covalently bound to residual peptides (U. Lindahl, unpublished work).

Attempts to purify the heparin preparations showed that extraneous protein could be separated simply by dissolving the freeze-dried materials in concentrated salt solutions. The insoluble residue thus obtained on treatment of mastocytoma heparin with 2M-KCl was recovered and subjected to amino acid analysis (Table 1). The results indicated that 81% (not corrected for moisture) of the isolated contaminant was protein. Hexosamine was absent. In a similar fashion a protein-rich material was isolated from a preparation of liver capsule heparin; the small amount of hexosamine present (Table 1) might be due to contamination with polysaccharide.

The mechanism of the interaction between CPC and the isolated proteins is unknown. Since the proteins contain large amounts of aspartic acid and glutamic acid (Table 1) it is conceivable that binding of cetylpyridinium ions to carboxyl groups may be of some significance. Denaturation induced by CPC leads to precipitation of protein; however, irreversibly denatured proteins would have been eliminated when CPC-precipitable material insoluble in 2M-KCl was removed by filtration through Celite. Also, the effect of high salt concentrations remains unclear, since protein isolated by precipitation with 2M-KCl had apparently survived similar conditions during the preceding part of the preparation.

As shown in Table 1 the two protein preparations isolated by precipitation with CPC from extracts of bovine liver capsule and mouse mastocytoma display marked similarities with respect to amino acid composition. Further, the results are in fair agreement with amino acid analyses of proteoglycans isolated both with and without the use of CPC (Gregory, Laurent & Rodén, 1964; Rosenberg, Schubert & Sandson, 1967; Serafini-Fracassini, Peters & Floreani, 1967; Serafini-Fracassini, Durward & Floreani, 1969). Apparently such analyses should be interpreted with considerable caution; they become meaningful only if one can show that the polypeptide material present is indeed covalently bound to the polysaccharide.

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